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Studies on the Folding of Heat-denatured Ovalbumin

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CONTENTS

INTRODUCTION	1
CHAPTER 1. Temperature Control for Kinetic Refolding of Heat-denatured Ovalbumin.	2
CHAPTER 2. Linear Polymerization Caused by the Defective Folding of a Non-inhibitory Serpin Ovalbumin.	75
CHAPTER 3. Phosphoryl and Carbohydrate Moieties in Ovalbumin have a Caperone-like Role of Preventing Aggregation.	121
SUMMARY	143
ACKNOWLEDGEMENT	147

ABBREVIATIONS

ANS, 1-anilinonaphthalene-8-sulfonate
BiP, immunoglobulin heavy chain binding protein
BPTI, bovine pancreatic trypsin inhibitor
BSA, bovine serum albumin;
CD, circular dichroism
DTT, dithiothreitol
DPCC, diphenylcarbonyl chloride
EDC, <i>N</i> -ethyl- <i>N'</i> -((3-di-methylamino)propyl)-carbodiimide hydrochloride
erfc, error function complement
GdmCl, guanidinium hydrochloride;
HPLC, high performance liquid chromatography
IAEDANS; <i>N</i> -iodoacetyl- <i>N'</i> -(5-sulfo-1-naphthyl)ethylenediamine
NHS, <i>N</i> -hydroxysuccinimide
NMR, nuclear magnetic resonance
PAGE, polyacrylamide gel electrophoresis
PAI-1, plasminogen activator inhibitor 1
PEI, polyethyleneimine
PMSF, phenylmethylsulfonyl fluoride
PVDF, polyvinylidene difluoride; RU, resonance unit
SDS, sodium dodecyl sulfate
TEM, transmission electron microscopy
TLC, thin layer chromatography

INTRODUCTION

Heating process can change the food in physical property, nutritive qualities and preservation. These changes are related to the structural changes of each protein molecules and association among denatured molecules in heating and cooling period, but poorly understood in molecular level. It make difficult to study the phenomenon which occur among the protein molecules in heating process that some reactions: the denaturation in heating, correct and incorrect refolding in cooling, association among denatured protein, have proceeded in the same sample.

From the experiment with ribonuclease Anfinsen had presented the hypothesis that protein fold spontaneously in the thermodynamic process. This hypothesis was supported in the folding experiment with small size proteins such as α -lactalbumin and cytochrome *c*. On the other hand, how Anfinsen's hypothesis could apply to large size proteins?

Recently it was reported that the folding process of a member of mammalian serine protease inhibitors (serpin), plasminogen activator inhibitor I, was controlled in kinetic. In this study, since ovalbumin is a relatively large and globular protein with the molecular size of 45 kDa and belongs to the serpin family, I studied on the folding from heat-denatured state and the association mechanisms between ovalbumin molecules of misfolded intermediates.

CHAPTER 1

Temperature Control for Kinetic Refolding

of Heat-denatured Ovalbumin

The folding of proteins has been generally considered to be under thermodynamic control since Anfinsen (1973) stated for the first time that the tertiary structure that is required for a protein to exert its biological activity is determined by the primary linear sequence of amino acids. Folding pathways for small proteins, such as α -lactalbumin and apomyoglobin, are thought to follow the framework model, which assumes a unique folding pathway with obligatory and sequential intermediates, according to the established hierarchy of the native structure (Kim & Baldwin, 1982, 1990; Ptitsyn, 1987; Matthews, 1993). Introduction of the concept of a molten globule state (Ohgushi & Wada, 1983; Kuwajima, 1989, 1992; Goto *et al.*, 1990; Christensen & Pain, 1991; Ptitsyn, 1992; Fink, 1995) has led to further probing of the intermediates in the framework model. The equilibrium molten globule state observed at acidic pH is generally defined as a partially folded compact state with a sizable hydrophobic core and a

significant native-like secondary structure but little of a persistent tertiary structure. Earlier studies showed that the equilibrium molten globule corresponds to kinetic intermediates in the folding of α -lactalbumin (Ikeguchi *et al.*, 1986; Kuwajima *et al.*, 1987) and of apomyoglobin (Hughson *et al.*, 1990; Barrick & Baldwin, 1993; Jennings & Wright, 1993). These findings have provided a link between the equilibrium molten globule and the kinetic intermediate in protein folding, and they lend strong support to the framework model.

Support is lent to another folding model wherein multiple folding pathways exist in the absence of a simple specific pathway and a series of unique intermediates (Wetlaufer, 1973; Konishi *et al.*, 1982). Recently Sosnick *et al.* (1994) demonstrated, by pulsed hydrogen exchange NMR, that the folding of oxidized cytochrome *c* can proceed very rapidly when the formation of non-native heme ligation is prevented, either by protonation of histidine residues or by addition of an extrinsic ligand, such as imidazole. For the correct folding of cytochrome *c*, a non-native interaction serves as a kinetic trap that allows accumulation of a partially misfolded intermediate and prevents the rapid formation of the native structure (Sosnick *et al.*, 1994; Elöve *et al.*, 1994). The occurrence of a kinetic trap in the folding of cytochrome *c* is explicitly mirrored by the

observations by NMR that revealed at least three successive kinetic phases (Roder *et al.*, 1988). Similar complex kinetics, with two or more successive phases, have been observed in the folding of other proteins, such as ribonuclease A (Udgaonkar & Baldwin, 1990), hen lysozyme (Radford *et al.*, 1992; Dobson *et al.*, 1994; Itzhaki *et al.*, 1994), and barnase (Bycroft *et al.*, 1990; Matouschek *et al.*, 1992a, b).

Multiple kinetic phases arise from the conformational heterogeneity of an unfolded protein at the beginning of its refolding. It seems likely that non-native interactions, as exemplified by *cis-trans* isomerization about prolyl peptide bonds and incorrect disulfide pairing, can function to promote the development of conformational heterogeneity in an unfolded state and to prevent rapid folding. Such a hypothesis serves to underlie the possibility that folding of a protein can be controlled by a kinetic process and it suggests, moreover, that a large protein with a more complicated structure might not always refold rapidly and efficiently, unlike small proteins such as α -lactalbumin and apomyoglobin do, because the larger protein contains many more proline and cysteine residues with increasing probability as the polypeptide chain increases in length. Recent findings suggest that the intermediates characterized to date can, perhaps, be considered to be

partially misfolded species that have been trapped kinetically by barriers that are not intrinsic to protein folding (Baldwin, 1995; Radford & Dobson, 1995).

Ovalbumin, a medium-sized glycoprophosphoprotein with a molecular size of 45 kDa, is the major globular protein of chicken egg white. It is a member of the serpin superfamily that includes mammalian PAI-1 and α_1 -antitrypsin, proteins that inhibit the serine proteases that regulate coagulation and fibrinolysis (Huber & Carrell, 1989). Ovalbumin has no such inhibitory activity (Long & Williamson, 1980; Ødum, 1987) despite sequence homology to antitrypsin of about 30%, but it acts as a substrate for elastase (Wright, 1984). As shown in Figure 1A, the crystal structure of native ovalbumin indicates that a five-stranded β -sheet runs parallel to the long axis of the molecule and an α -helix protrudes as a loop that forms the reactive center (Stein *et al.*, 1991). It seems likely that molecular structures of the active PAI-1 and α_1 -antitrypsin correspond to that of the native ovalbumin. PAI-1 and α_1 -antitrypsin molecules easily undergo structural changes. PAI-1 is synthesized in a form that is active as an inhibitor but is relatively unstable. This active form changes slowly to a form that is more thermostable but is inactive as an inhibitor. This latter form is known as the latent form (Franke *et al.*, 1990). If the latent form is unfolded in a denaturant or at a high temperature, the unfolded PAI-

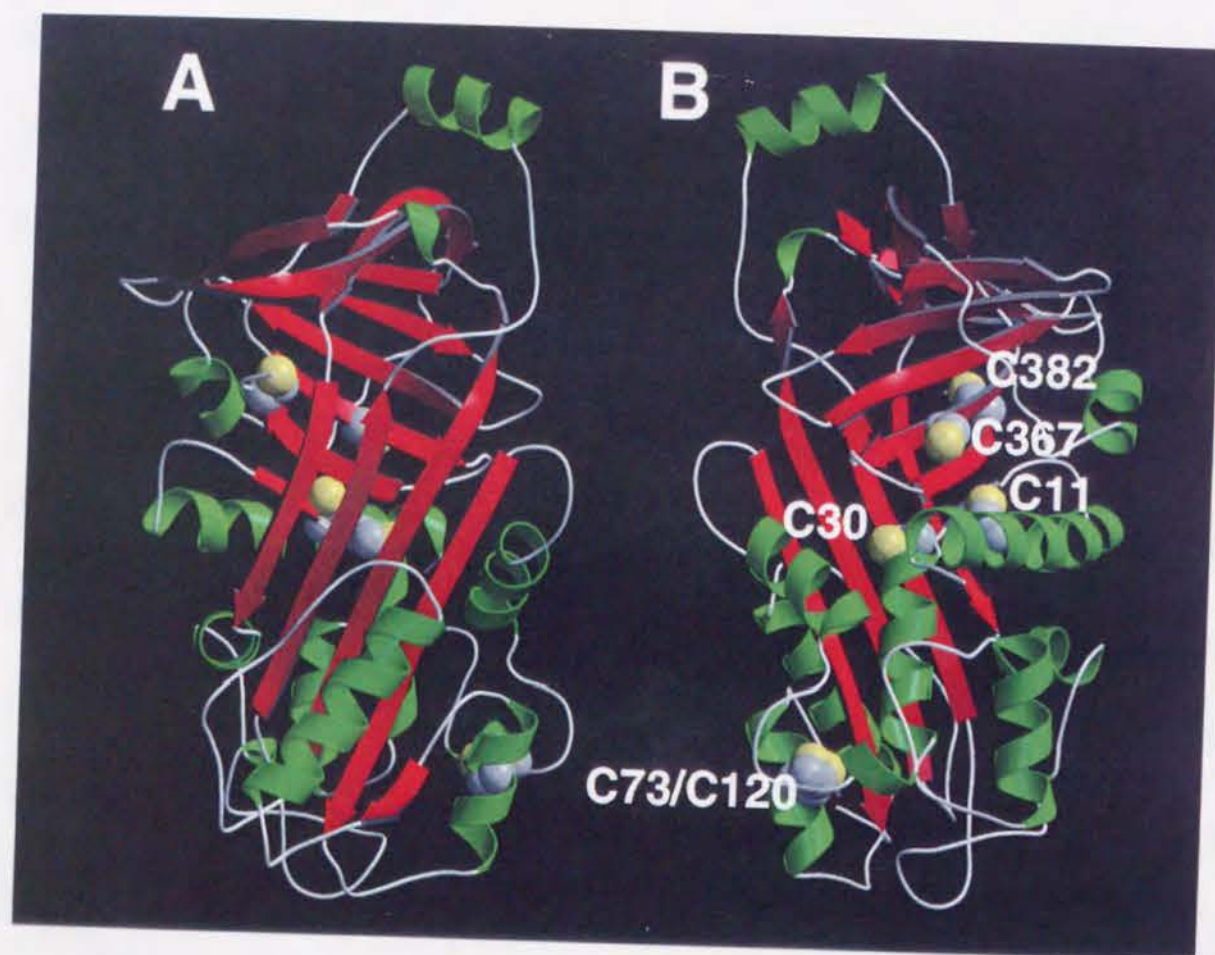


Figure 1. Schematic representation of the structure of hen egg-white ovalbumin (Stein *et al.*, 1991). α -Helices are represented by green helical ribbons and strands of β -sheets by red arrows. Sulfur atoms, and both α - and β -carbon atoms in cysteine residues are shown as yellow and gray spheres, respectively. (A) Front view. The central five-stranded β -sheet, running parallel to the long axis of the molecule, and the α -helix protruding from the molecule are postulated to be unique structural characteristics of ovalbumin in the serpin superfamily. (B) Back view. Cysteine residues are identified by C, with numbers that show their positions in the primary structure. The diagram was drawn using the programs MOLSCRIPT (Kraulis, 1991) and Raster3D Ver. 2.0 (Merritt & Murphy, 1994).

1 refolds to generate the active form which again changes slowly but spontaneously to the latent form (Hekman & Loskutoff, 1985; Katagiri *et al.*, 1988). In this conversion, some residues in the loop that forms the reactive center are instead poised to form the central strand of a six-stranded β -sheet (Mottonen *et al.*, 1992). α 1-Antitrypsin undergoes an irreversible conformational change upon cleavage at the reactive-center loop to assume a more thermostable configuration (Loebermann *et al.*, 1984), in which part of the reactive-center loop is inserted as an additional strand into the β -sheet. Moreover, Powell and Pain (1992) found that chemically denatured α 1-antitrypsin refolds into a compact non-native form which is different from the native conformation and, furthermore, that the non-native form with molten globule characteristics is able to fold slowly into the native form. These various phenomena indicate that the active conformation of a serpin is determined by a kinetically controlled process (Creighton, 1992; Baker & Agard, 1994).

Another noteworthy feature of ovalbumin is the presence of a single disulfide bond and four free cysteine sulfhydryl groups. A disulfide bond connects Cys73 and Cys120 in a solvent-accessible environment, while four cysteine sulfhydryl groups are buried within the intramolecular hydrophobic core (Figure 1B). It has been reported that ovalbumin fails to reassume its native conformation when it is

allowed to renature from a fully denatured state (Simpson & Kauzmann, 1953; Imahori, 1960). Klausner *et al.* (1983) observed that the renaturation of chemically denatured ovalbumin results in the formation of a stable but altered form of the protein. The altered form has hydrophobic characteristics that distinguish it from the native protein. Under non-reducing and denaturing conditions, a rapid thiol-disulfide exchange occurs if a protein has both thiol and disulfide moieties (Creighton, 1986). With this exchange in mind, Takahashi and Hirose (1992) demonstrated that correct oxidative renaturation occurs effectively from denatured and disulfide-reduced ovalbumin at a low concentration of the protein but not from disulfide-bonded ovalbumin. If the non-native disulfide bonds interfere with correct refolding, it seems probable that a kinetically trapped intermediate accumulates during the folding of ovalbumin under non-reducing conditions. Thus, we can speculate that, under specific conditions, the folding of ovalbumin might be determined by kinetic rather than thermodynamic parameters.

The folding kinetics of a protein can be determined in terms of a folding rate, which depends predominantly on the external environment, such as the temperature and the solvent (Matthews, 1993). When heated at 80°C in the presence of salt, ovalbumin produces a highly ordered linear polymer, as does α_1 -

antitrypsin (Koseki *et al.*, 1989; Stein & Carrell, 1995). From CD spectra, intrinsic tryptophan fluorescence and surface hydrophobicity, the authors found that ovalbumin molecules in a chain polymer adopt a molten globule structure and that polymerization can be controlled by manipulating the protein concentration and the ionic strength of the medium (Tani *et al.*, 1995).

In the present study, the authors examined the effects on the correct refolding of heat-denatured ovalbumin by manipulating the folding temperature. The results show that manipulation of the folding temperature allowed the folding of heat-denatured ovalbumin to follow either of two pathways: one pathway toward the native state and one pathway toward the intermediate with molten globule characteristics. The intermediate accumulated as a large variety of compact conformations that were trapped in a kinetically accessible state as a result of non-native disulfide interactions. Since the intermediate was slowly but spontaneously transformed to the native form, the correct refolding of heat-denatured ovalbumin was eventually achieved by both alternative pathways. Thus, the folding of ovalbumin can be determined by kinetic controls, according to a given set of circumstances. The folding behavior conforms to theoretical expectations that are based on statistical mechanics and this behavior can be described by a rugged energy landscape.

Materials and Methods

Materials

Ovalbumin was purified from the egg white of newly laid hens' eggs by crystallization in a solution of ammonium sulfate and it was recrystallized five times (Sørensen & Höyrup, 1915). Trypsin, soybean trypsin inhibitor (type I-S) and chymotrypsin (type II) were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin (type XI; Sigma) that had been treated with DPCC was used for digestion of the refolded ovalbumin, while another preparation of trypsin (type III; Sigma) was used for fragmentation of labeled ovalbumin for determination of the arrangement of disulfide bonds. Bovine serum albumin (type I), carbonic anhydrase and whale skeletal muscle myoglobin (type II) were also obtained from Sigma. From Aldrich Chemical Co. (Milwaukee, WI) was purchased IAEDANS. *Achromobacter* protease I (EC 3.4.21.50) and ANS were obtained from Wako Pure Chemical Industries (Osaka, Japan). Iodoacetoamide, guanidinium chloride and urea of specially prepared reagent grade and other chemicals of guaranteed grade were obtained from Nacalai Tesque (Kyoto, Japan).

Methods

Refolding of heat-denatured ovalbumin

A solution of ovalbumin at 0.25 mg ml⁻¹, containing 0.01 mM EDTA, was adjusted to pH 7.5 with 0.1 N NaOH and a pH meter (Toko Chemical Lab. Co., Ltd., Tokyo, Japan). After adjustment of the pH, an aliquot of the solution was heated at 80°C for 5 min in a borosilicate glass tube. Slow cooling of the heat-denatured ovalbumin was achieved by gradually decreasing the temperature from 80°C to 0°C at a rate of 0.45°C min⁻¹ (model RTE-110; NESLAB, Newington, NH). Rapid cooling was achieved by quick transfer of the glass tube containing the protein that had been heat-denatured at 80°C to an ice-water bath. In kinetic studies, an aliquot of the rapidly-cooled product was incubated at the indicated temperatures for various periods of time.

Proteolytic digestion

Digestion with trypsin was performed as described by Takahashi and Hirose (1992). A solution of refolded ovalbumin (0.25 mg ml⁻¹) was buffered with 0.025 volume of 1 M Tris-HCl (pH 8), mixed with 12.5 mg ml⁻¹ DPCC-treated trypsin at a ratio of protease to ovalbumin of 2.5 : 1 (w/w) and the mixture was incubated at 30°C for 1 min. The digestion was terminated by the addition of soybean trypsin inhibitor, in an amount of that was twice of the protease. The samples were subjected to electrophoresis on an SDS-polyacrylamide gel by the standard method

described by Laemmli (1970) and stained with Coomassie Brilliant Blue R-250. Aliquots equivalent to 2.5 μg of ovalbumin were loaded in each lane for SDS-PAGE. The amounts of trypsin-resistant species were evaluated from the intensities of bands, which were measured with a densitometer (CS-910; Shimadzu, Kyoto, Japan). The yield of renatured protein is expressed as the amount of the trypsin-resistant species that remained as a percentage of the non-digested protein.

Size-exclusion chromatography

The aggregation of heat-denatured ovalbumin was analyzed on a column of TSKgel G3000SWXL (7.8 i.d. x 300 mm; Tosoh, Tokyo, Japan). The column was equilibrated with 20 mM sodium phosphate buffer (pH 7.5), and the samples were eluted with the same buffer at a flow rate of 0.5 ml min⁻¹. Ovalbumin (0.25 mg ml⁻¹) was heated at 80°C for the periods indicated, in the absence or presence of various concentrations of sodium phosphate buffer (pH 7.5) that contained 0.01 mM EDTA. Heated samples were applied onto the column after cooling in an ice-water bath. The amount of ovalbumin applied onto the column was 20 μg . The percentage of monomeric ovalbumin was determined from the ratio of the peak area of the monomer to those of the total protein.

Stokes radii of the native and the compactly misfolded ovalbumin were estimated with a column of TSKgel G2000SWXL (7.8 i.d. x 300 mm; Tosoh) that had been equilibrated with 20 mM sodium phosphate buffer (pH 7.5). Chromatography was performed as described above. Molecular Stokes radii of the species of ovalbumin were estimated from experimentally determined elution volumes by the calibration method of Ackers (1967). The void volume was based on the elution volume of blue dextran. The total solvent-accessible column volume was based on the elution volume of sodium azide.

Measurements of circular dichroism

CD spectra were recorded with a spectropolarimeter (model J-720; Jasco, Tokyo, Japan) equipped with an interface and a computer. The temperature was controlled with a cell holder that was thermostatically controlled (model RTE-110; NESLAB).

The unfolding transition curve for ovalbumin was obtained by measuring the ellipticity at 222 nm at a protein concentration of 0.1 mg ml⁻¹ in a 1-mm cell. The temperature was increased from 25°C to 85°C at a rate of 1.0°C min⁻¹ for the heat-denaturation process. The change in temperature was monitored with a thermocouple (type SK-2000MC; Sato Keiryoki Mfg. Co., Ltd., Japan) and a

flexible probe inserted directly into the 1-mm cell. The measured ellipticities were corrected for the monitored temperatures with a computer.

Far-UV CD spectra were recorded at a protein concentration of 0.25 mg ml⁻¹ with a 1-mm cell at wavelengths from 250 to 195 nm. The data were collected three times and expressed as the average mean residue ellipticity. Near-UV CD spectra were recorded at a protein concentration of 0.25 mg ml⁻¹ with a 2-cm cell at wavelengths from 245 to 310 nm. The data were collected three or five times and expressed as molar ellipticity.

Temperature-jump experiments were performed by transferring manually ovalbumin that had been heat-denatured at 80°C for 5 min into a 1-mm cell that was thermostatically maintained at the indicated temperature. The protein concentration was 0.25 mg ml⁻¹. Refolding was monitored for 1 hr by measuring the change in ellipticity at 222 nm in the spectropolarimeter.

Measurements of fluorescence

Tryptophan fluorescence spectra were recorded at 25°C except in the case of the heat-denatured ovalbumin, and they were recorded with a fluorescence spectrophotometer (F-3000; Hitachi, Tokyo, Japan). The concentration of the sample was 1 μM. The excitation wavelength was 295 nm and the emission

wavelength ranged 305 to 400 nm.

For measurements of ANS fluorescence, various species of ovalbumin molecules were incubated at 25°C for 5 min with a 20-fold molar excess of ANS at a protein concentration of 1 μM. The excitation wavelength was 390 nm and the emission wavelength was 470 nm. Emission spectra were corrected for background fluorescence caused by ANS in reaction mixtures prepared without the protein.

Analysis of disulfide bonds

Procedures were those developed by Tatsumi *et al.* (1994) unless otherwise specified. In brief, a solution of urea and HCl was added to a solution of ovalbumin at a protein concentration of 0.25 mg ml⁻¹ to give final concentrations of 9 M urea and 0.25 M HCl, for complete unfolding of the polypeptide chain and to avoid further rearrangement of disulfide bonds. After incubation at 37°C for 5 min, the acid-quenched protein was vigorously mixed with 0.57 volumes of a solution of 1.1 M Tris base that contained 0.4 M iodoacetamide and 9 M urea to give a final pH of 8.8, and alkylation of protein sulfhydryl groups was achieved by incubation at 37°C for 10 min. The protein was precipitated with a mixture of cold acetone and 1 N HCl (98:2, v/v), washed three times with a mixture of cold acetone, 1 N HCl and H₂O (98:2:10, v/v), dissolved in buffer [50 mM Tris (pH 8.8), 1 mM EDTA] that

contained 9 M urea to give a protein concentration of 1.6 mg ml⁻¹, and then fully reduced by incubation with 5 mM DTT at 37°C for 30 min. The disulfide-reduced ovalbumin was labeled by incubation at 37°C for 10 min with 15 mM IAEDANS. The labeled protein was precipitated with acetone-HCl, washed as described above, and extensively digested by two-step proteolysis. The first step involved digestion with trypsin (1/25, w/w; type III; Sigma) and chymotrypsin (1/50, w/w; type II; Sigma) at 30°C for 20 hrs, and the second step involved incubation with chymotrypsin (1/50, w/w; type II; Sigma) and *Achromobacter* protease I (1/100, w/w; Wako) at 30°C for 20 hrs.

When the disulfide bonds in ovalbumin that had been heat-denatured at 80°C were analyzed, HCl was added during the heating at 80°C to a final concentration of 10 mM. Immediately after the addition of HCl, the protein solution at 80°C was quickly cooled in an ice-water bath. The acidified protein solution was treated with a solution of urea and HCl as described above. For use as the standard sample, native ovalbumin was fully reduced and unfolded at 37°C for 30 min in the presence of buffer that contained 9 M urea and 5 mM DTT. After incubation, disulfide-reduced ovalbumin was incubated at 37°C for 10 min with 15 mM IAEDANS.

Digested samples of ovalbumin were applied to a reverse-phase HPLC column (4.6 x 150 mm; Cosmosil 5C18-AR; Nacalai Tesque) connected to an HPLC system (LC-10AS; Shimadzu) equipped with a spectrofluorometric detector (RF-550; Shimadzu). The elution pattern of labeled peptides in a standard sample was the same as those reported by Tatsumi *et al.* (1994). The relative amount of a disulfide-involved half-cystine residue was estimated from the areas of peaks of fluorescent material for each half-cystine in the sample and the standard.

Kinetic analysis

To estimate the rapid-phase amplitude and to calculate the folding rate during the slow phase, k_{slow} , the ellipticity observed over the course of 1 hr in a temperature-jump experiment was fitted to the following equation:

$$y(t) = A + \sum B_i (1 - \exp(-t / \tau_i)),$$

where $y(t)$ is the amplitude of the ellipticity at 222 nm at time t , A is the amplitude at zero time, B_i is the maximal amplitude corresponding to each phase, i is the phase number, and τ_i is the associated relaxation time corresponding to each phase for the refolding of the protein. A two-state transition mechanism was approximated to the refolding at 75°C for the heat-denatured and the refolded

states. Biphasic kinetics were introduced for folding at temperatures below 75°C. The folding rate during the slow phase, k_{slow} , was estimated as the reciprocal of the relaxation time during the slow phase.

Results

Heat-denaturation process

Effects of ionic strength on the polymerization of ovalbumin

Figure 2 shows the profiles of elution after gel-permeation chromatography, demonstrating the polymerization of denatured molecules that occurred when ovalbumin was heated at pH 7.5 at a concentration of 0.25 mg ml⁻¹ under various conditions related to ionic strength and the duration of heating. Ovalbumin polymerized when it was heated at 80°C even for a mere 5 min in the presence of 20 mM phosphate buffer (Figure 2A). However, the polymerization depended on the concentration of phosphate buffer (Figure 2B, C). In addition, the polymerization depended critically on the duration of incubation at 80°C: a peak of material with a retention time indistinguishable from that of native ovalbumin increased in height with decreases in the duration of heating. These observations suggested that polymerization could be inhibited primarily by a decrease in both the ionic strength of the medium and the duration of heating. Indeed, when the medium was adjusted to pH 7.5 with NaOH in the absence of salt, more than 99% of ovalbumin was estimated to be in a monomeric form after incubation at 80°C for

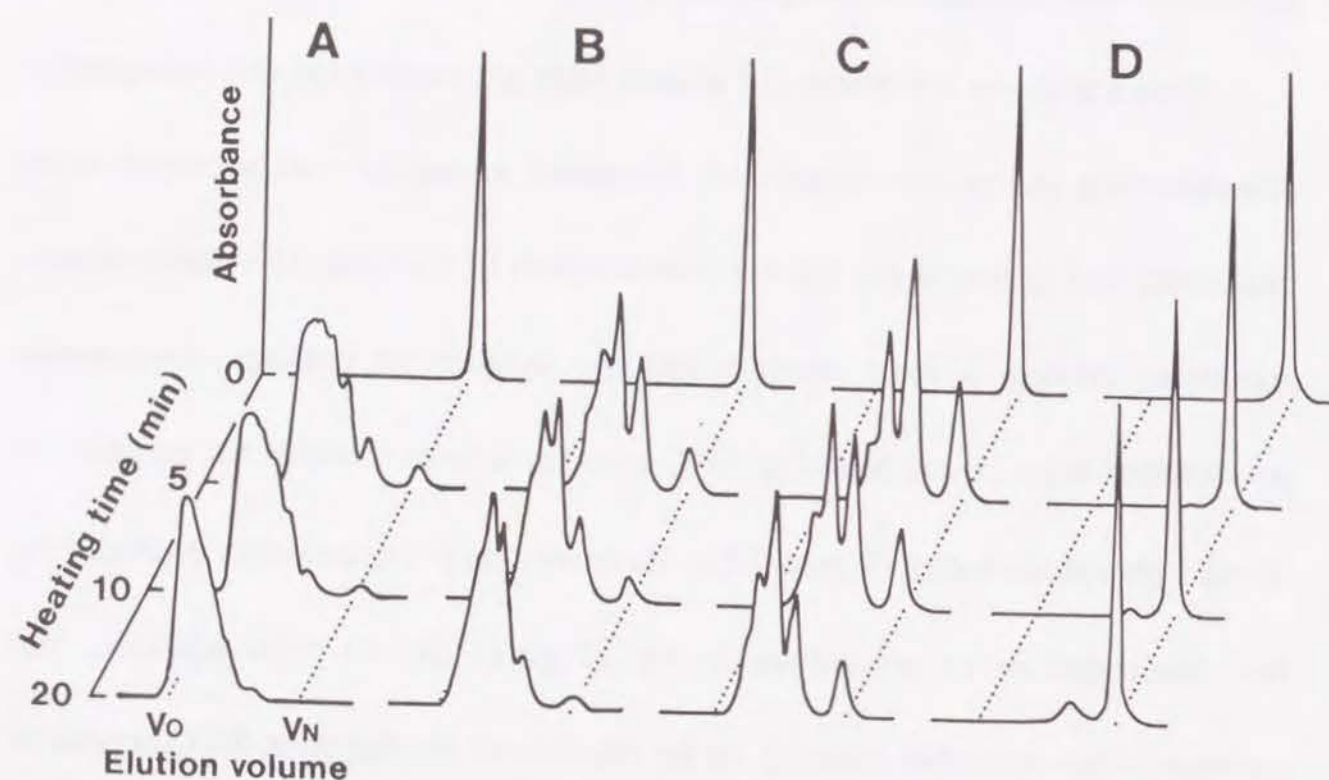


Figure 2. A comparison of the elution profiles of variously treated samples of ovalbumin during size-exclusion chromatography. Ovalbumin was heated at 80°C for the times indicated in the presence of sodium phosphate buffer (pH 7.5) at 20 mM (A), 10 mM (B), 5 mM (C), or in the absence of buffer (D). V_0 and V_N indicate the void volume and the elution volume of native ovalbumin, respectively. Absorbance at 280 nm is in arbitrary units.

5 min, whereas approximately 4% of the ovalbumin formed putative dimers after incubation for 20 min. This finding indicates that ovalbumin can undergo heat-induced unfolding and refolding in a monomeric state in a process that is controlled by the ionic strength and the duration of heating.

Thermal unfolding of ovalbumin in a monomeric state

The author examined the heat-induced unfolding of ovalbumin by CD. Figure 3 shows the change in the ellipticity at 222 nm of intact ovalbumin under non-reducing conditions at pH 7.5. Evidence of a highly cooperative unfolding was obtained. It seems likely that the unfolding can be approximated by a mechanism for a two-state transition between the folded and unfolded states with a midpoint temperature of 76°C. It should be noted that the ovalbumin molecule unfolds almost completely at 80°C. The absolute ellipticity at 222 nm was greater at 80°C than that of the protein that had been unfolded by 6 M GdmCl, an observation that suggests that thermally denatured ovalbumin does not behave as an ideal random-coiled chain.

Alternative cooling process

Susceptibility to protease of the folded states upon cooling

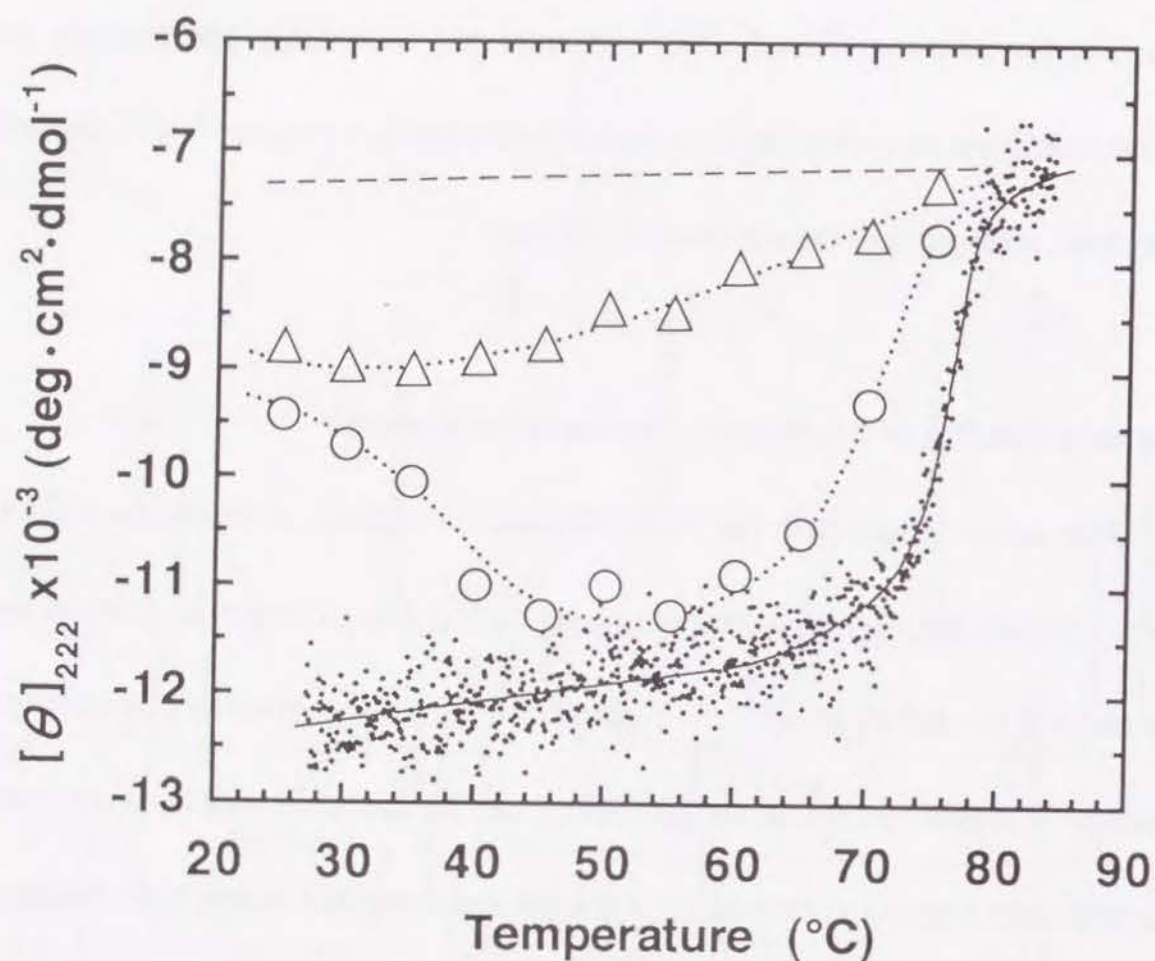


Figure 3. Temperature-dependent unfolding of ovalbumin. The observed ellipticity at 222 nm is shown by dots. The temperature was increased from 25°C to 85°C at a rate of 1.0°C min⁻¹ for the heat-denaturation process. Open circles show the final ellipticity expected from the temperature-jump refolding. Open triangles show the ellipticity observed after the rapid phase of ovalbumin folding.

One of the sensitive and accurate methods that have been established for examining the conformation of ovalbumin is analysis by tryptic digestion (Takahashi & Hirose, 1992). The principle on which this optimized method is based is that native ovalbumin is completely resistant to digestion by trypsin whereas heat-denatured or acid-treated ovalbumin is prone to this digestion (Ottesen & Wallevik, 1968). The author used this simple method to examine the conformation of heat-denatured ovalbumin after slow cooling (Figure 4A-I) and rapid cooling in ice-water bath (Figure 4A-II). These cooled products were hereafter referred to as the slowly-cooled and the rapidly-cooled products, respectively.

When the heat-denatured ovalbumin was cooled slowly, the trypsin-resistance was recovered in a high yield. As shown in Figure 4B-a, the recovery was greater than 85% at a cooling rate of 0.45°C min⁻¹. This result indicated that heat denaturation was reversed by slow cooling in the case of thermally unfolded ovalbumin. By contrast, a low yield of less than 10% of the trypsin-resistance was obtained when the heat-denatured ovalbumin was cooled rapidly in an ice-water bath (Figure 4B-b), suggesting that the heat-denatured ovalbumin had folded to generate an alternate non-native conformation that was susceptible to trypsin. The striking difference in yield between the slow and rapid cooling processes indicates

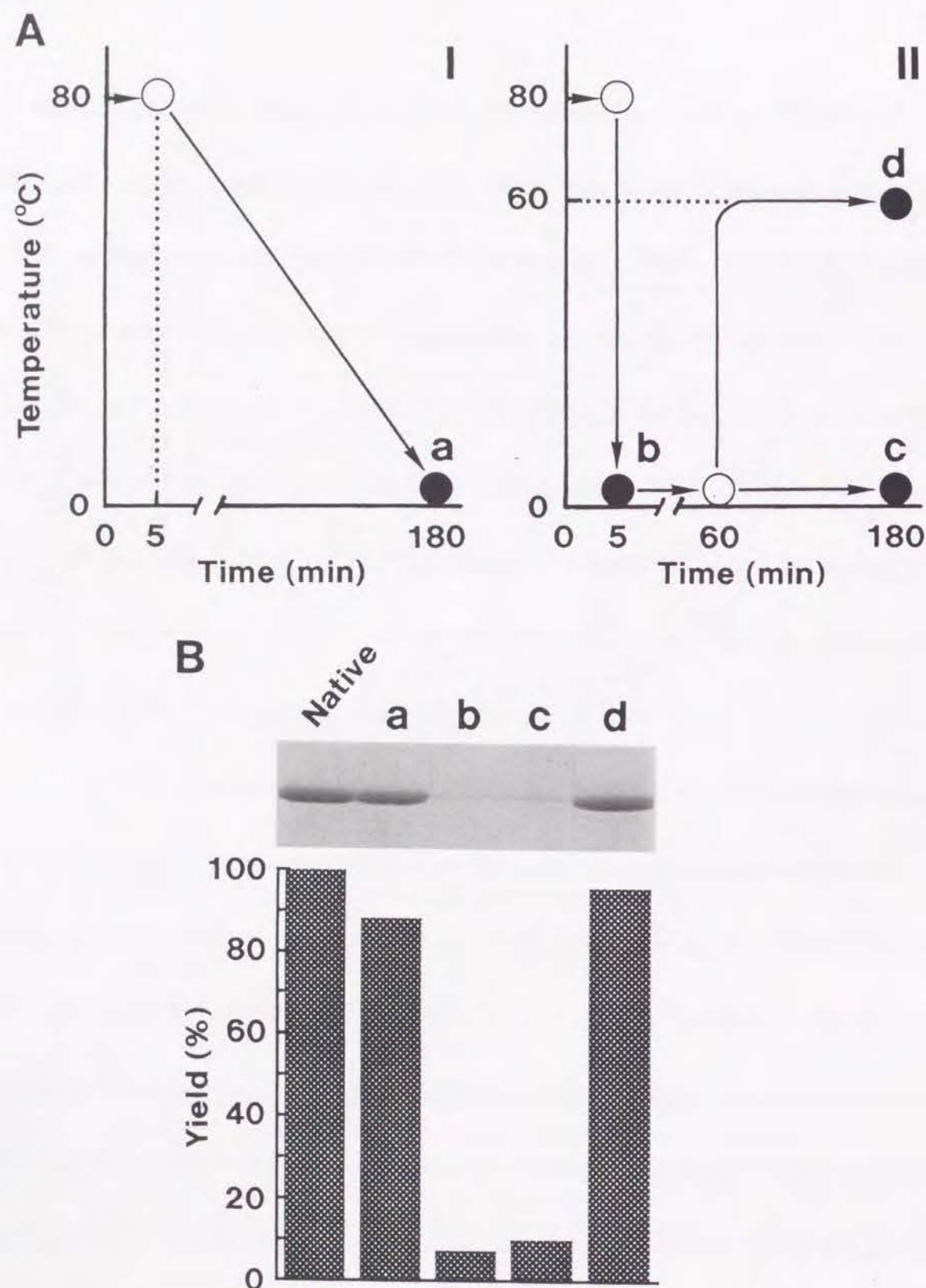


Figure 4. Formation of ovalbumin species that are resistant to trypsin digestion by alternative cooling processes. (A) Diagrams illustrating the treatments of heat-denatured ovalbumin. (B) Yield of trypsin-resistant species of ovalbumin at each point indicated in A. An aliquot of the sample of ovalbumin was analyzed for susceptibility to digestion by trypsin at the points indicated by closed circles.

that the refolding of heat-denatured ovalbumin depends to a significant extent on the cooling rate.

Thermal stability of the folded states

Figure 4 provides further information about the folding of heat-denatured ovalbumin. During prolonged storage in an ice-water bath, the rapidly-cooled product remained unaltered as a trypsin-sensitive species (Figure 4B-c). To our surprise, incubation at 60°C for 2 hr of the rapidly-cooled product (Figure 4A-II) provoked conversion of the trypsin-sensitive to the species that was resistant to the proteolytic digestion, with a yield of more than 85% (Figure 4B-d).

Figure 5A shows the dependence on temperature of the transformation of the rapidly-cooled product into the trypsin-resistant species. As might be expected, the rapidly-cooled product remained unchanged at 4°C. An increase in the incubation temperature accelerated the rate of acquisition of resistance to the proteolytic digestion. The times required for transformation of half of the rapidly-cooled product were approximately 3 hr and 30 min at incubation temperatures of 37°C and 60°C, respectively. Maximal transformation was achieved by incubation at 60°C for two to three hours. The yield of the trypsin-resistant species was reduced

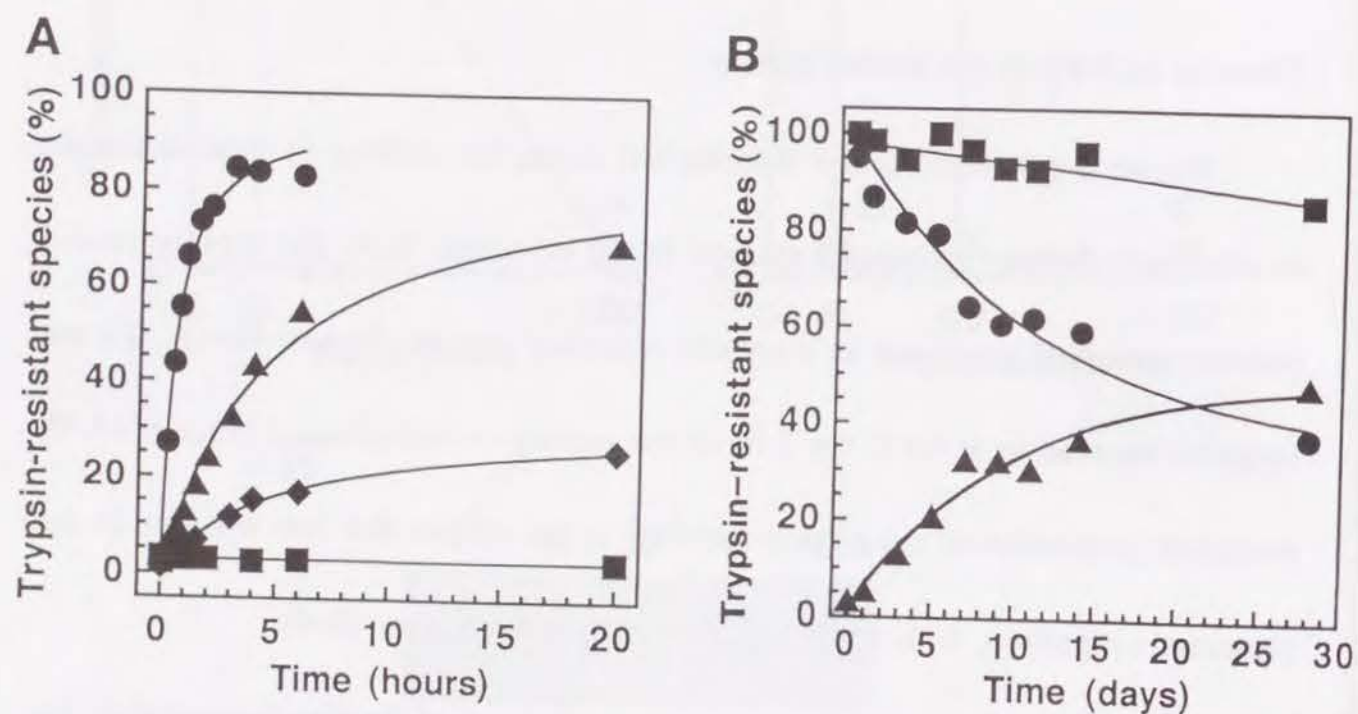


Figure 5. Thermal stability of the folded ovalbumin. (A) Temperature-dependent conversion of rapidly-cooled product to trypsin-resistant species. The rapidly-cooled product was incubated at the indicated temperature: 60°C (circles), 37°C (triangles), 25°C (diamonds), and 4°C (squares) for the periods indicated. (B) Stability of rapidly-cooled product at a low temperature. After the storage of the rapidly-cooled product at 4°C for the number of days indicated, the amount of the trypsin-resistant species was examined before or after the incubation at 60°C for 2 hrs. The former was estimated as spontaneously renatured molecules (triangles), and the latter as totally renatured molecules. The amount of non-native species remaining at a low temperature (circles) was evaluated as the difference between the former and the latter. The percentage of the monomeric protein (squares) was determined by size-exclusion chromatography.

by prolonged incubation at 60°C, perhaps as a result of the formation of non-specific aggregates.

Figure 5B shows the stability of the rapidly-cooled product at an ice-water bath temperature over the course of several weeks. Gel-permeation chromatography revealed that a large portion of the rapidly-cooled product remained monomeric for four weeks, even though there was a gradual loss of monomeric ovalbumin. The loss of the monomer probably resulted from the formation of the putative dimer as a consequence of hydrophobic interactions. It should be noted that, as time elapsed, some of the trypsin-sensitive monomers slowly but spontaneously acquired resistance to proteolytic digestion, even when stored at a low temperature. About three weeks were required to convert 50% of the protein to trypsin-resistant species. The remaining monomers were converted to a protease-resistant species by incubation at 60°C. These results provide evidence that the ovalbumin species produced by the rapid cooling process were somewhat stable but slowly underwent conformational changes, being in a metastable state as compared to the native state because the native protein undergoes no conformational changes up to 65°C (see Figure 3).

Characterization of structures

Compactness

Stokes radii of refolded species of ovalbumin were evaluated by size-exclusion chromatography on a column of TSKgel G2000SWXL, which was able to separate proteins with molecular sizes from 10^4 to 10^5 kDa with a satisfactory high resolution. The ovalbumin that had been refolded by a slow cooling process was eluted at the same retention time as that of the native protein. Its Stokes radius was estimated to be 28.0 Å. By contrast, the ovalbumin species trapped by rapid cooling was eluted at the retention time that was slightly different from that of the native protein (Figure 6A). Its Stokes radius was estimated to be 29.6 Å, approximately 6% larger than that of the native protein (Figure 6B). However, the Stokes radius was substantially smaller than the calculated value of 62.0 Å for the ovalbumin completely unfolded in 6 M GdmCl (Corbett & Roche, 1984). These data suggest that the ovalbumin species obtained on rapid cooling is compactly misfolded in an alternate state that is distinguishable from the native state. The trypsin-resistant species produced by incubation at 60°C of the rapidly-cooled product had the same Stokes radius as that of the native protein (data not shown).

Secondary structure

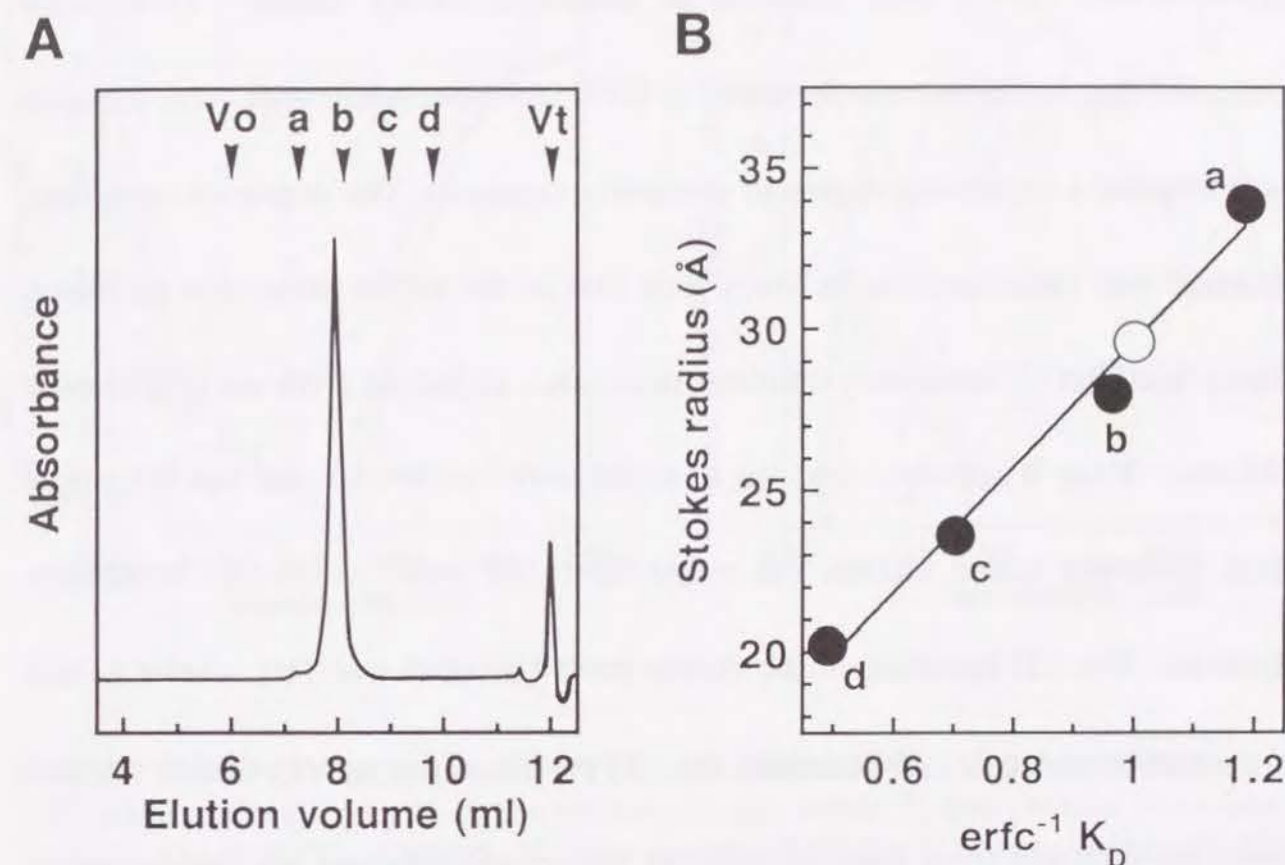


Figure 6. Elution profile of the rapidly-cooled product during size-exclusion chromatography (A) and the relationship between the Stokes radius and $\text{erfc}^{-1} K_D$ of the globular proteins (B). The proteins used for calibration were (a) bovine serum albumin (67 kDa), (b) ovalbumin (45 kDa), (c) bovine carbonic anhydrase (30 kDa), and (d) whale skeletal muscle myoglobin (16.9 kDa). V_0 and V_t represent the void volume and the total solvent-accessible column volume, respectively. Closed and open circles indicate the relationships in the proteins used for calibration and the rapidly-cooled product, respectively.

Panel A in Figure 7 shows the CD spectra in the far-UV region. Ovalbumin molecules that had been denatured by GdmCl did not have an ordered secondary structure but, rather, they behaved as randomly coiled chains. Ovalbumin molecules that had been heat-denatured at 80°C in a monomeric state were found to have retained a significant degree of secondary structure. The degree of secondary structure was considered to be lower than that of the native molecules but much higher than that of chemically unfolded molecules, as judged from the ellipticity at 222 nm. Thus, it appeared that the heat-denatured molecules did not behave as ideal randomly coiled chains, but might have had some native-like secondary elements. The CD spectrum of the slowly-cooled product was very similar to that of the native molecule. By contrast, the CD profile of the rapidly-cooled product was quite different from those of both the native molecule and the slowly-cooled product. Nonetheless, it was fairly similar to that of the heat-denatured molecules, and such similarity suggests that the rapidly-cooled product possesses secondary structural elements that are distinguishable from the native-like elements. Incubation at 60°C of the rapidly-cooled product converted its CD profile to that of the native molecule.

Tertiary structure

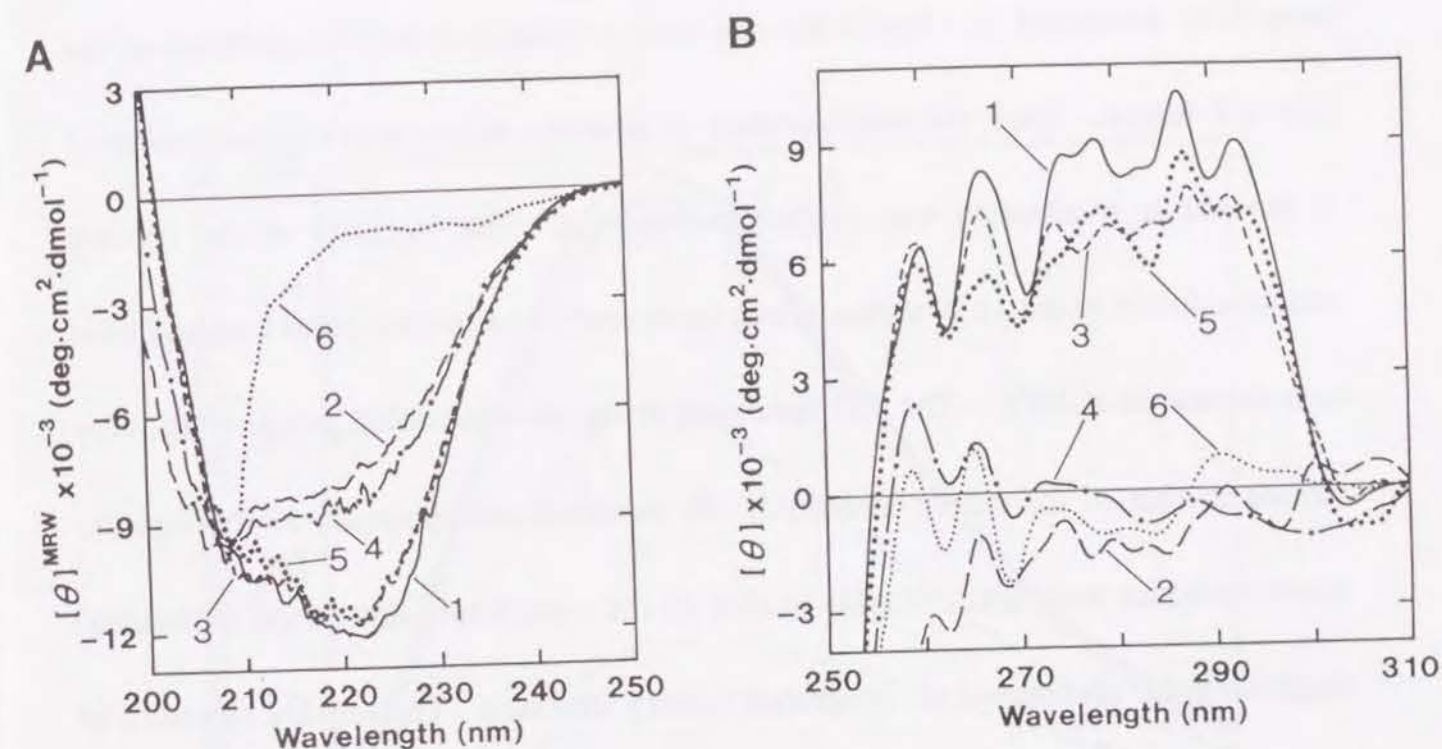


Figure 7. Far-UV (A) and near-UV (B) CD spectra of different forms of the ovalbumin molecule: curve 1, the native protein; curve 2, the protein after heat-denaturation at 80°C; curve 3, the slowly-cooled product; curve 4, the rapidly-cooled product; curve 5, the thermally-transformed product; curve 6, the protein that had unfolded completely in the presence of 6 M GdmCl.

Panel B in Figure 7 shows the near-UV CD spectra. The molecule that had been fully denatured by GdmCl did not have a characteristic CD spectrum in the near-UV region. Thus, the configuration of aromatic amino acid residues assumed in the native ovalbumin was probably disrupted. The integrity of the tertiary structure found in native ovalbumin was completely lost in molecules that had been heat-denatured at 80°C. The CD spectrum of the slowly-cooled product was very similar to that of the native molecule. By contrast, the spectrum of the rapidly-cooled product was rather similar to that of the completely denatured molecules, suggesting the absence of an integrated tertiary structure. The tertiary structure of molecules that had been thermally transformed from the rapidly-cooled product appeared to have been reconstructed to yield native-like architecture.

Fluctuation

The changes in the microenvironment around tryptophan residues were examined by monitoring the intrinsic fluorescence of the, native, heat denatured, slowly cooled, rapidly cooled, thermally transformed and chemically denatured states (Figure 8). A fluorescence emission maximum was found at 336 nm for the native ovalbumin. When ovalbumin was treated with GdmCl, the emission maximum shifted to 352 nm, with a resultant 64% decrease in the relative

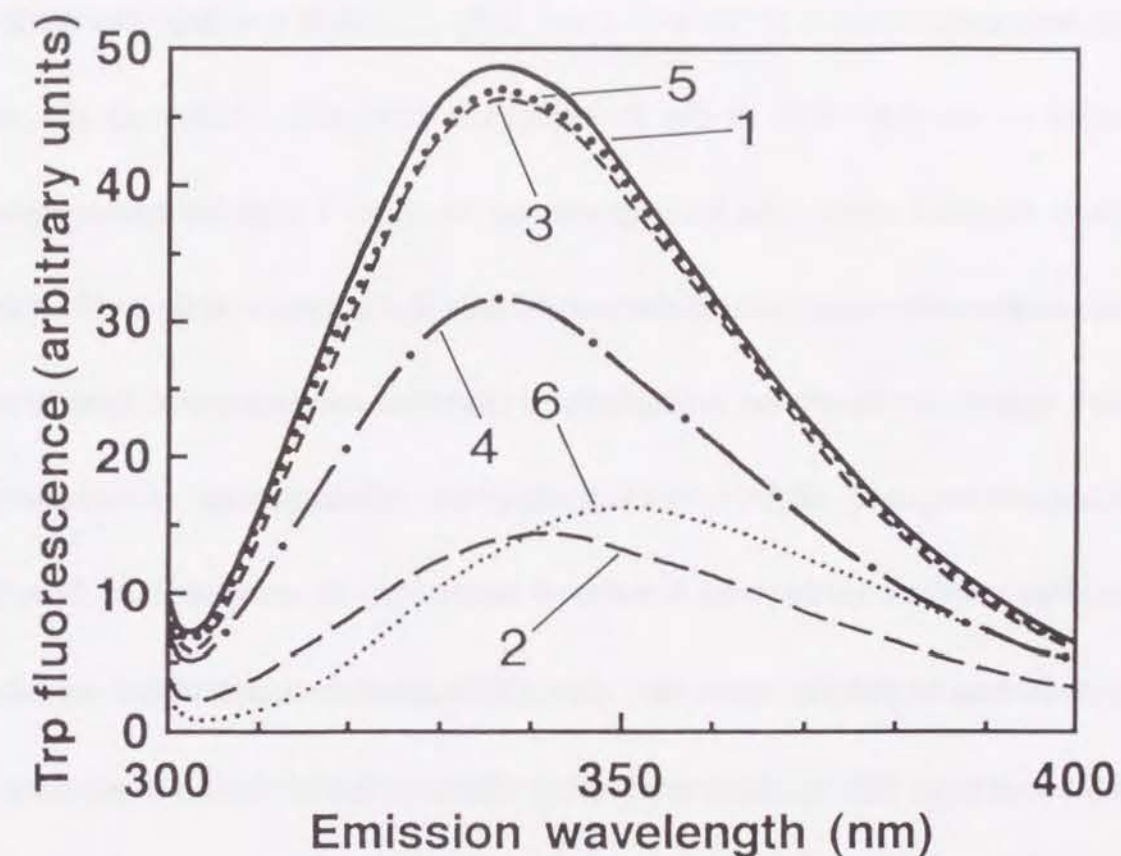


Figure 8. Tryptophan fluorescence emission spectra of different forms of the ovalbumin molecule: curve 1, the native protein; curve 2, the protein after heat-denaturation at 80°C; curve 3, the slowly-cooled product; curve 4, the rapidly-cooled product; curve 5, the thermally-transformed product; curve 6, the protein that had unfolded completely in the presence of 6 M GdmCl. The numbers on the left denote arbitrary units.

fluorescence intensity. The fluorescence changed markedly when the protein had been heated at 80°C; the emission maximum was 342 nm, about halfway between the fluorescence maxima of the native and fully unfolded proteins, and there was a decrease of roughly 70% in the fluorescence intensity. Although the relative intensity required correction for comparison because it depended to a significant extent on the temperature, a red shift of 40% in the emission reflects the fact that, relative to those in the native protein, the tryptophan residues in the heat-denatured molecules were in a slightly more hydrophilic environment. Conversely, the tryptophan residues in the heat-denatured molecules, as compared to those in the GdmCl-denatured protein, were in a more hydrophobic environment, as indicated by a blue shift of 60% in the emission. A fluorescence emission maximum of the slowly-cooled product was observed at 336 nm, at the same wavelength as the fluorescence maximum of the native protein gives, with almost the same relative fluorescence intensity. The rapidly-cooled product had the same emission maximum, at 336 nm, as the native protein has, but the relative intensity was intermediate between those of the native and the chemically denatured molecules. The blue shift in the emission, relative to the chemically denatured and heat-denatured molecules, implies that the tryptophan residues in the rapidly-cooled product is buried within a hydrophobic environment similar to those residues in the

native protein. The relative fluorescence intensity of the thermally-transformed product was the same as that of the native protein, without any shift in the fluorescence maximum.

Surface hydrophobicity

The surface hydrophobicity of various conformations of ovalbumin was measured with a chromophore, ANS, as the probe, as shown in Figure 9. The native ovalbumin did not exhibit any fluorescence of ANS. The protein denatured by GdmCl exhibited no fluorescence because hydrophobic clusters cannot be formed in a random-coiled state. Similarly, the molecules that had been heat-denatured at 80°C exhibited little fluorescence. This result indicates that the hydrophobic residues might not cluster together in the heat-denatured molecules because of large fluctuations, regardless of the dependence on temperature of the fluorescence intensity. The slowly-cooled product did not exhibit any fluorescence, resembling the native protein. By contrast, the rapidly-cooled product exhibited significant fluorescence, a result that suggests the exposure of some hydrophobic clusters at the surface of the protein. These results are complementary to the observation that, in the rapidly-cooled product, the sulfhydryl reagent IAEDANS reacted rapidly with exposed sulfhydryl groups located within those sequences rich

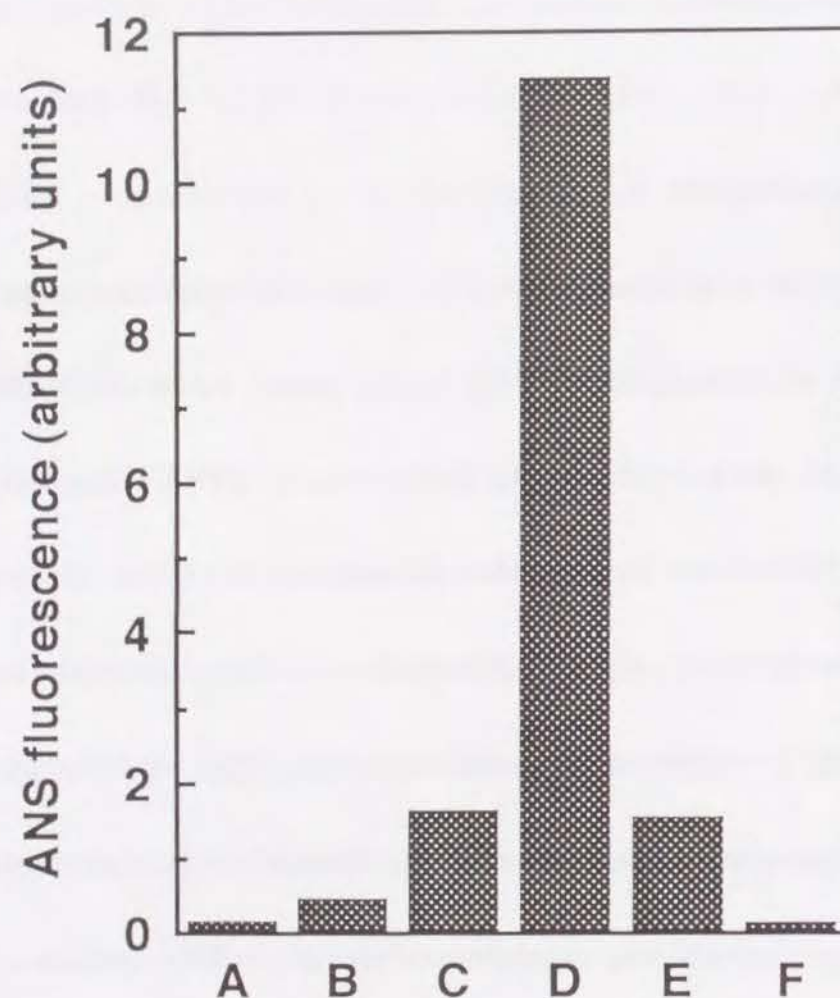


Figure 9. ANS fluorescence of different forms of the ovalbumin molecule: A, the native protein; B, the protein after heat-denaturation at 80°C; C, the slowly-cooled product; D, the rapidly-cooled product; E, the thermally-transformed product; F, the protein that had unfolded completely in the presence of 6 M GdmCl. The numbers on the left denote arbitrary units.

in hydrophobic amino acids. The strong fluorescence observed with the rapidly-cooled product was markedly quenched by its conversion to the thermally-transformed product.

Disulfide bonds

Figure 10 shows the labeling patterns by IAEDANS used for the location of cysteine residues that were involved in the formation of a disulfide bond. Only two cysteine residues, Cys73 (SH3) and Cys120 (SH4), which form the native disulfide bond, were labeled in native ovalbumin (Panel A). By contrast, six cysteine residues were all labeled after ovalbumin had been heat-denatured at 80°C (Panel B). This suggests that heat-denatured ovalbumin is a heterogeneous mixture of various disulfide isomers. The high values obtained in this analysis for both SH5 and SH6 could reflect the formation of the entropy-favored shortest disulfide loop. As shown in panel C, the profile for the slowly-cooled product was the same as that for the native protein. By contrast, the profile for the rapidly-cooled product (Panel D) qualitatively matches that for heat-denatured molecules, although the extent of labeling of both SH5 and SH6 appears to be slightly reduced. The thermally-transformed product had two labeled cysteine residues, SH3 and SH4, as did both the native molecule and the slowly-cooled product (Panel E). Chemically

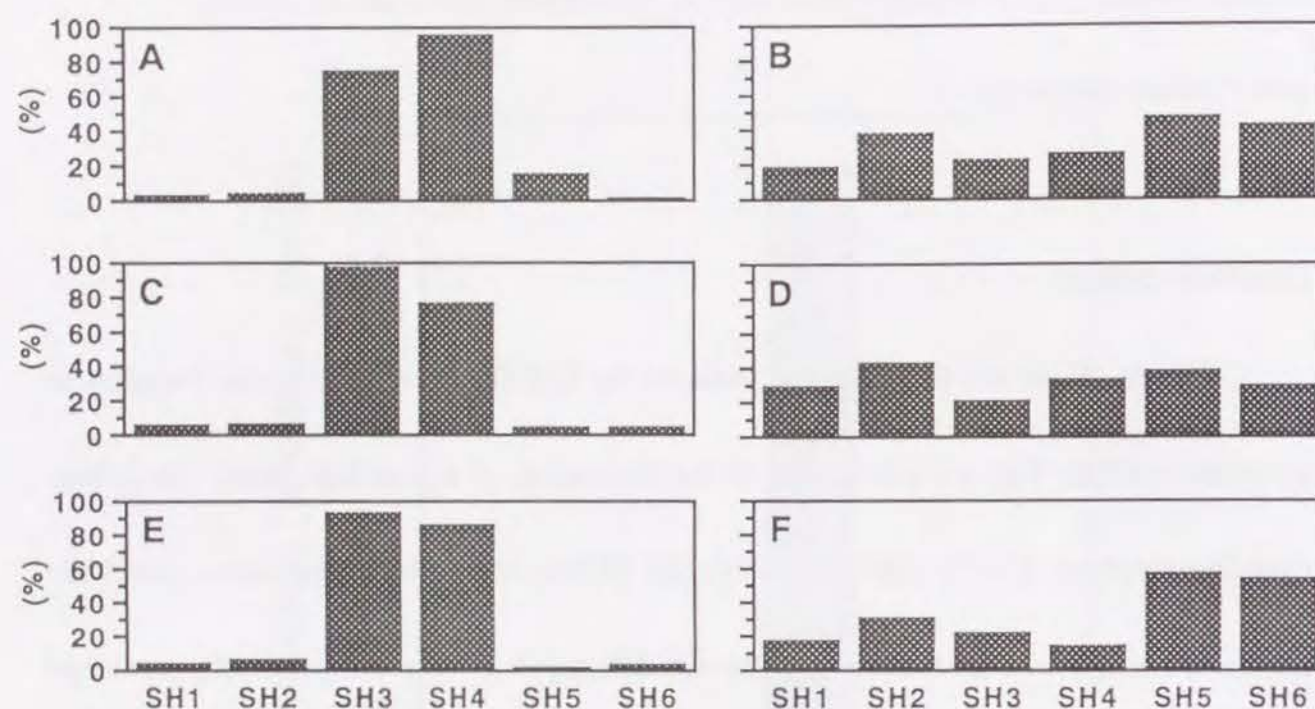


Figure 10. Distribution of cysteine residues that are involved in disulfide bond formation after labeling with a fluorescent dye: A, the native protein; B, the protein after heat-denaturation at 80°C; C, the slowly-cooled product; D, the rapidly-cooled product; E, the thermally-transformed product; F, the protein that had unfolded completely in the presence of 6 M GdmCl. SH1, SH2, SH3, SH4, SH5, and SH6 refer to Cys11, Cys30, Cys73, Cys120, Cys367, and Cys382, respectively.

unfolded ovalbumin (Panel F) had six labeled cysteine residues. Formation of entropy-favored short disulfide loops was observed in the following order: Cys367 (SH5) and Cys382 (SH6); Cys11 (SH1) and Cys30 (SH2); and Cys73 (SH3) and Cys120 (SH4), as reported by Tatsumi *et al.* (1994).

Folding kinetics

Biphasic kinetics during temperature-jump refolding

Figure 11 shows the changes in the ellipticity at 222 nm when the folding of heat-denatured ovalbumin was allowed to proceed at the indicated temperatures. The ellipticity remained unaltered at the temperature of 80°C (Panel A). Folding of heat-denatured ovalbumin was observed when the temperature was set below 75°C. At 75°C, the change in ellipticity could be fitted to a curve following first-order kinetics. The decrease in the ellipticity after refolding for 1 hr became larger as the temperature was lowered from 70°C (Panel B) to 60°C (Panel C). The maximum decrease was observed at a temperature between 60°C and 50°C. However, the change in ellipticity decreased at a temperature below 50°C (Panel D-F). It is noteworthy that, below 70°C, the curves tended to deviate from those expected from monophasic first-order kinetics and to suggest biphasic kinetics for rapid and slow phases. A remarkable feature was that the ellipticity decreased

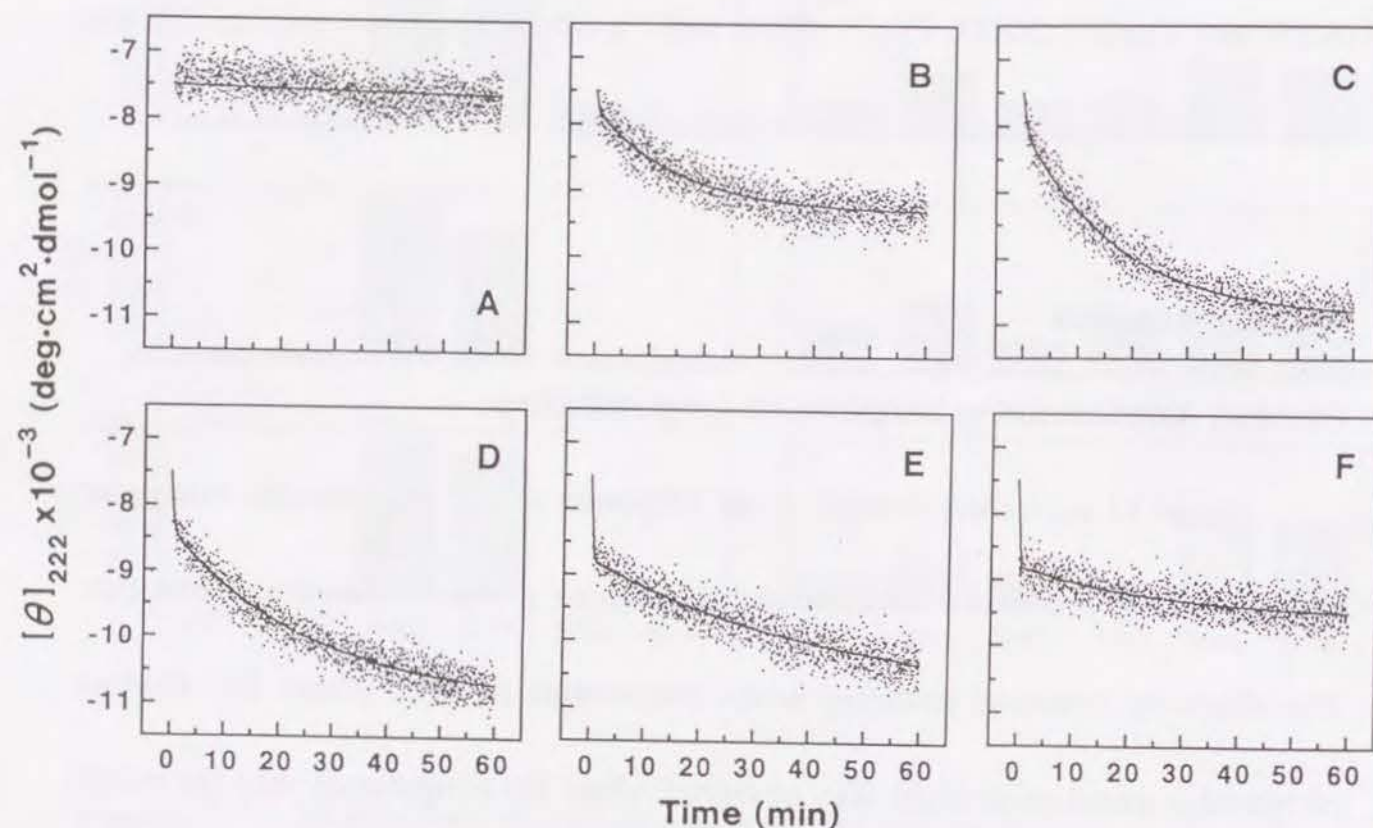


Figure 11. Temperature-dependent changes in the ellipticity at 222 nm of the ovalbumin molecule. Ovalbumin that had been heat-denatured at 80°C was transferred to a thermostatically controlled 1-mm cell that was maintained at the temperature indicated: A, 80°C; B, 70°C; C, 60°C; D, 50°C; E, 40°C; F, 30°C. The ellipticity observed at 222 nm is shown by dots. Each line is the curve that corresponds most closely to the equation given in Materials and Methods.

within the dead time required for manual mixing, and that its amplitude increased as the temperature was lowered. The rapid phase was followed by the slow phase and its amplitude decreased gradually with decreases in temperature. At 30°C, the ellipticity after the rapid phase suggested almost the same amount of secondary structure as that of the non-native species formed by rapid cooling.

Yield of the renatured protein

Figure 12 shows the resistance to proteolytic digestion of ovalbumin molecules that had been allowed to fold for 1 hr at the indicated temperatures. The yield of trypsin-resistant species increased as the folding temperature was lowered over the range from 80°C to 55°C. The maximum yield was achieved at 55°C. A further decrease in the folding temperature below 55°C resulted in a decreased yield.

Discussion

Conformational features of various species of ovalbumin

The heat-denatured state

The denatured state of a protein should play a crucial role in determining its folding and stability. For the definition of the pathway and kinetics of folding of

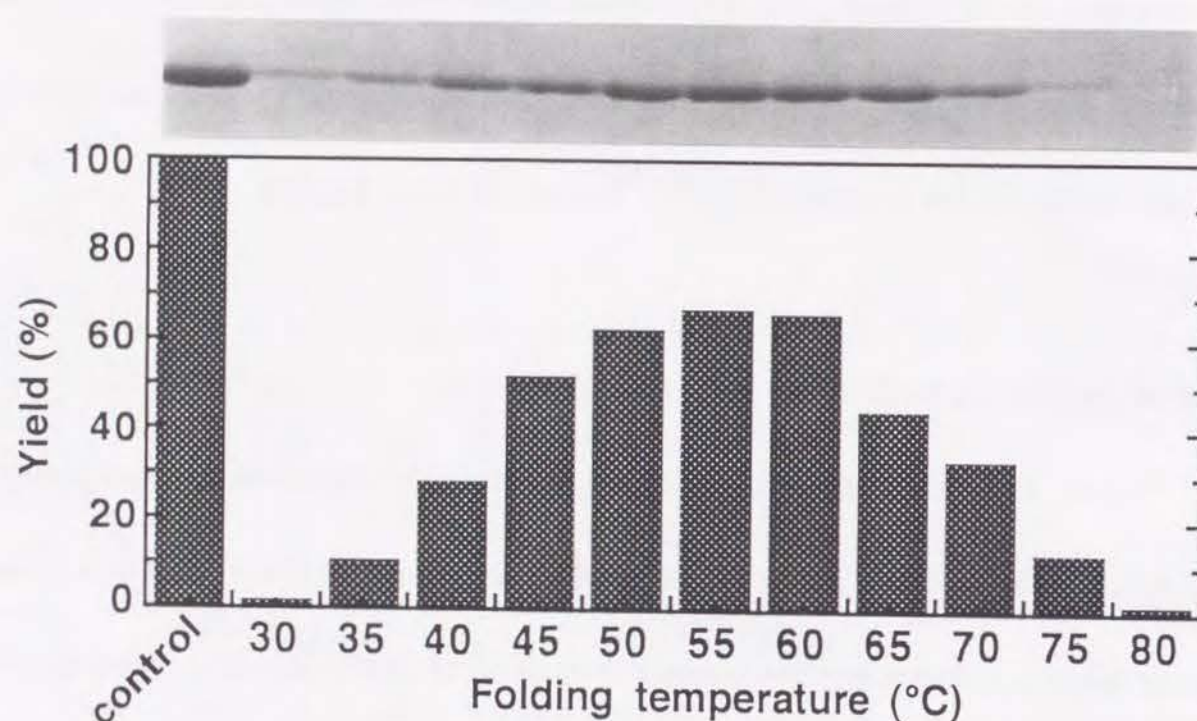


Figure 12. Yields of renatured protein after the refolding of heat-denatured ovalbumin for 1 hr at the folding temperatures indicated.

heat-denatured ovalbumin, it is necessary to characterize the way in which the polypeptide chain unfolds under heated conditions and to identify the non-native interactions that occur at the beginning of refolding.

The CD spectra showed that the heat-denatured ovalbumin had substantial secondary structure but lacked a persistent tertiary structure with highly mobile side-chains (Figure 7). These features seem to satisfy the criteria for the molten globule state. Some proteins, such as α -lactalbumin (Dolgikh *et al.*, 1981) and RNase A (Labhardt, 1982), display significant CD in the far-UV region under thermally denatured conditions. The cited reports also indicate that residual secondary structure might exist in the heat-denatured state. The intrinsic fluorescence of tryptophan revealed unique characteristics of the heat-denatured ovalbumin (Figure 8). The native ovalbumin has three tryptophan residues within the intramolecular hydrophobic core, as indicated by the emission maximum of 336 nm. By contrast, in the chemically denatured ovalbumin, these residues were completely exposed in a solvent-accessible environment, as indicated by the red shift in the emission to 352 nm. The emission maximum of the heat-denatured ovalbumin was 342 nm, being intermediate between values for the native and the completely unfolded molecules. This result demonstrates that the intramolecular

hydrophobic core becomes less condensed and more accessible to the solvent molecules. However, some hydrophobic interactions might remain intact, unlike those in the chemically unfolded protein in a random-coiled state. Our observations are also consistent with the previous indication that, in thermally denatured hen lysozyme, side chains on aromatic residues cluster together even at temperatures as high as 81°C (Evans, 1991).

On heat-denaturation of ovalbumin, non-native interactions occur and thiol-disulfide exchanges proceed extensively, as judged from our observation that six cysteine residues were all labeled with a fluorescent reagent albeit to varying extents (Figure 10). Such exchange was not due to artificial oxidation because it was not suppressed by further addition of chelator and dispersion of dissolved oxygen by argon. This observation suggests that the polypeptide backbone fluctuates considerably during heating at 80°C. However, SH5 and SH6 appear to be more heavily labeled in the chemically unfolded ovalbumin than in the heat-denatured ovalbumin, showing that an entropy-favored short loop is easily generated in the chemically unfolded protein. It seems likely that the freedom of movement of the polypeptide chain in the heat-denatured state might be somewhat restricted as a result of residual hydrophobic interactions, as compared to the chemically unfolded protein.

Methods involving NMR have been increasingly applied to the structural analysis of the non-native states of proteins. Hydrogen-exchange NMR was used to demonstrate large structural fluctuations in the heat-denatured protein, and no stable hydrogen-bonded structure was detected in a thermally denatured preparation of RNase A (Robertson & Baldwin, 1991). Recently, solution X-ray scattering has provided useful information about the size and shape of proteins in the molten globule state (Nishii *et al.*, 1994; Kataoka *et al.*, 1995). Application of this technique demonstrated that heat-denatured ovalbumin was more expanded than native molecules but less expanded than chemically unfolded molecules (unpublished data). Several lines of evidence suggest that heat-denatured ovalbumin does not behave as an ideal randomly coiled chain as is observed when a protein has been completely unfolded by chemical denaturants. The protein appears to assume an unfolded state with a large displacement of its polypeptide backbone, but with some degree of compactness, which is probably a consequence of hydrophobic interactions.

Alternatively folded states

Combining the results from analysis of disulfide bonds with the data from size-exclusion chromatography, measurements of CD and intrinsic tryptophan

fluorescence, and the adsorption of ANS (Figures 6 to 10), the author can deduce that the slowly-cooled and the thermally-transformed products, which both were resistant to trypsin, were equivalent to the native molecule. Results obtained by differential scanning calorimetry support this interpretation (data not shown). By contrast, the rapidly-cooled product, which was susceptible to trypsin, adopted a compact and misfolded conformation. This non-native state meets the criteria that have been widely used to define the molten globule state (Kuwajima, 1989; Baldwin, 1991). The rapidly-cooled product was condensed, with a Stokes radius slightly greater than that of the native protein but much smaller than that of the unfolded protein, with the persistence of secondary structural elements but lost tertiary structure, and with the exposure of some hydrophobic clusters to the solvent-accessible environment. However, our analysis of the rearrangement of disulfide bonds indicated that the rapidly-cooled product was a mixture of non-native disulfide isomers. The author can, therefore, consider the rapidly-cooled product to be a wide variety of compact conformations that collectively display the characteristics of the molten globule state.

Kinetic control of the folding of ovalbumin

Kinetic folding of heat-denatured ovalbumin

The striking contrast in the susceptibility to trypsin of the folded polypeptide obtained by either slow or rapid cooling of heat-denatured ovalbumin demonstrates that the folding of heat-denatured ovalbumin depends to a significant extent on the cooling rate, in other words, the folding rate. Slow cooling allows complete renaturation of heat-denatured ovalbumin. By contrast, rapid cooling forces the thermally unfolded molecules to misfold compactly and to adopt alternate non-native conformation, that are susceptible to trypsin. This finding implies that the folding of heat-denatured ovalbumin is controlled by a kinetic process.

It is generally believed that folding of a small protein is governed by a thermodynamic process. The thermodynamic hypothesis stated by Anfinsen holds that the native state of a protein is at a global free-energy minimum relative to all other states. Assuming that folding of a protein is under thermodynamic control, a two-state transition mechanism provides a good approximation of the conversion between the native and denatured states, and the mechanism should proceed along a single pathway between the unfolded and folded states. The amount of renatured protein would increase monotonically as the folding rate increases, as reported in the case of chymotrypsin inhibitor 2 from barley (Jackson & Fersht, 1991).

From our temperature-jump experiment (Figure 11), the author estimated the final ellipticity at 222 nm that would be expected if the folding reaction proceeds to

completion at a specific temperature (open circles in Figure 3). At folding temperatures between 80°C and 55°C, the expected ellipticities appeared to be close to the observed values on the unfolding transition curve although slight deviation was evident at temperatures between 70°C and 75°C. A folding transition curve as assessed from the degree in ellipticity exhibits a cooperativity (dotted line with open circles in Figure 3). The yield of renatured protein increased concomitantly with the decrease in the folding temperature from 80°C to 55°C (Figures 13A and 12). These results indicate that the folding of heat-denatured molecules can apparently be explained by a two-state transition mechanism, if molecules are cooled slowly enough to allow them to reach thermodynamic equilibrium. The slow cooling process that the other studied might reflect a folding reaction that is under thermodynamic control.

At folding temperatures below 55°C, the expected ellipticity deviated more and more from the observed values on the unfolding curve, with a slight increase in the rapid-phase amplitude as the folding temperature was lowered (Figure 3). It is noteworthy that the yield of renatured protein decreased significantly within this range of temperature, with dominance of the rapid-phase amplitude in the total change in ellipticity (Figure 13A) and with reduction in the folding rate in the slow phase (Figure 13B). This marked biphasic kinetics is inconsistent with the two-state

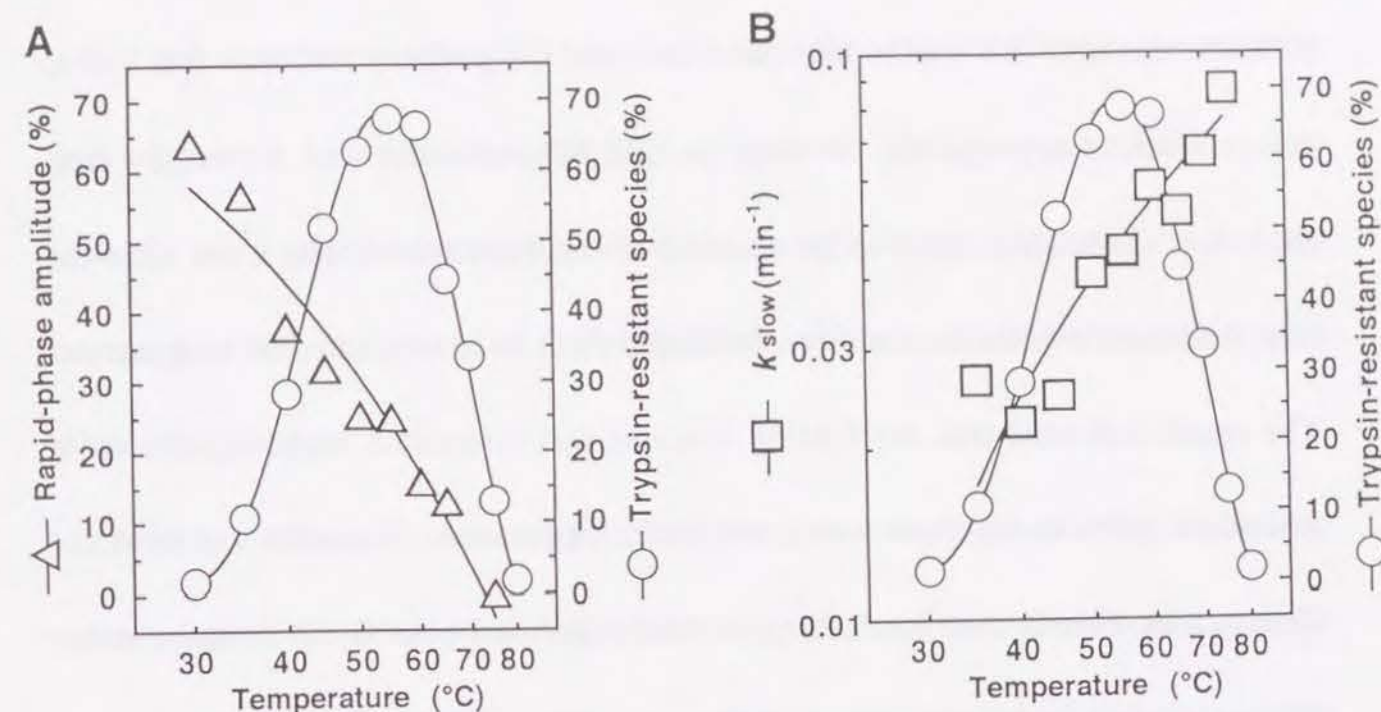


Figure 13. Relationships between the yield of renatured protein (circles) and the rapid-phase amplitude (triangles) (A) and the folding rate during the slow phase, k_{slow} , of heat-denatured ovalbumin (squares) (B).

transition mechanism. The rapid cooling process that the author studied can be regarded as a folding reaction that is under kinetic control.

Folding of a protein in general involves kinetic competition between on-pathway reactions that lead to the native state and off-pathway reactions that lead to non-productive aggregation. A large portion of ovalbumin was shown, by size-exclusion chromatography, to be retained in the monomeric state even after the heat-denatured ovalbumin had been incubated for 1 hr at the indicated temperature. The result indicated that the folding reaction was controlled under conditions in which off-pathway reactions were completely suppressed. These lines of evidence demonstrate clearly that the folding of heat-denatured ovalbumin is under either thermodynamic or kinetic control in the on-pathway reactions, depending on the environmental conditions.

Kinetic barriers to folding of ovalbumin

The rapidly-cooled product was able to refold to yield the native molecule slowly but spontaneously. Addition of thermal energy accelerated this transformation. To account for the slow transformation, it is necessary to invoke kinetic barriers between the rapidly cooled state and the native state.

Non-native disulfide bonds are known to interfere with the oxidative refolding of a small disulfide-containing protein such as BPTI (Creighton, 1988; Weissman & Kim, 1991, 1992). Oxidative folding of insulin-like growth factor 1 yields two disulfide isomers with similar thermodynamic stability, an observation that suggests a bifurcating protein-folding pathway (Miller *et al.*, 1993). One example of a marginally stable disulfide isomer being trapped as a kinetic intermediate was recognized in the study of the oxidative folding of human insulin (Hua *et al.*, 1995).

Many disulfide isomers existed under non-reducing and heat-denaturing conditions at 80°C (Figure 10B), although their distribution could not be quantitated accurately because heat-denatured molecules do not behave as ideal randomly coiled chains. However, the pattern of distribution remained unaltered on condensation of the thermally unfolded polypeptide chain by rapid cooling. This result suggests that correct rearrangement of disulfide bonds did not proceed during rapid cooling. Therefore, the kinetic barriers that retarded correct refolding can be interpreted in terms of non-native disulfide bonds that were preformed in the heat-denatured ovalbumin; non-native disulfide bonds directly disturb the search for a conformation with native folding. The reduction of a disulfide bonds relieves the strain, with the immediate acquisition of the native

conformation. This conclusion is consistent with the finding that some disulfide-reduced molecules of ovalbumin that have been unfolded by a denaturant can refold to yield the native conformation within a few minutes after the initiation of refolding, but the disulfide-bonded protein cannot (Takahashi & Hirose, 1992).

The author is unable, however, to exclude the possibility of interference by mechanisms other than formation of non-native disulfide bonds. A conformational trap, as well as non-native disulfide pairings, might play a subsidiary role in retarding refolding. The conformations of compactly misfolded molecules are presumed to fluctuate significantly in the molten globule state, and non-native pairing could easily be rearranged if a non-native disulfide bond had the chance to find itself close to a free thiol. The possible existence of a conformational trap is indicated by the observations of human α_1 -antitrypsin made by Powell and Pain (1992). Despite the fact that this serpin molecule contains only a single cysteine residue, the chemically unfolded protein refolded to a compact non-native form, different from the native conformation, and the non-native form that exhibited molten globule characteristics was able to fold slowly to the native form. These observations account for the existence of a conformationally trapped molecule during the refolding process.

Therefore, it appears that the kinetic barriers that retard the correct folding of ovalbumin can be attributed to both non-native disulfide pairings, for the most part if not entirely, and to conformational strain. It is of interest that the folding of ovalbumin is different from the oxidative refolding of small proteins such as BPTI and RNase A, in so far as folding of ovalbumin can be retarded by a combination of non-native disulfide bonds and a conformational trap.

A kinetic intermediate during folding

In the light of a slow conversion of the rapidly-cooled product to the native structure, the rapidly-cooled product can be regarded as a metastable intermediate that is trapped in a kinetically accessible state during the course of the refolding of heat-denatured ovalbumin. The characteristics of the intermediate meet many of the criteria commonly used to define a molten globule state. The intermediate state is separated from the native state by a substantial energy barrier. The intermediate also resembles the kinetically trapped intermediate in the refolding of α -lytic protease in some respects (Baker *et al.*, 1992), but it differs from the protease intermediate in that it is a heterogeneous population of non-native disulfide isomers that collectively display characteristics of a disordered molten globule state.

A marked contrast in heterogeneity between the kinetic and equilibrium intermediates supports the hypothesis that various molten globule states exist (Baldwin, 1991), which range from a heterogeneous population of molecules in the disordered molten-globule state to a homogeneous population of structured molecules in the molten globule state. Ovalbumin also adopts an equilibrium molten globule state under acidic conditions at pH 2 (Koseki *et al.*, 1988). The molecules in the equilibrium molten globule state are highly structured, and their population may be rather homogeneous because rearrangement of disulfide bonds does not occur readily as a consequence of the protonation of sulfhydryl groups under acidic conditions.

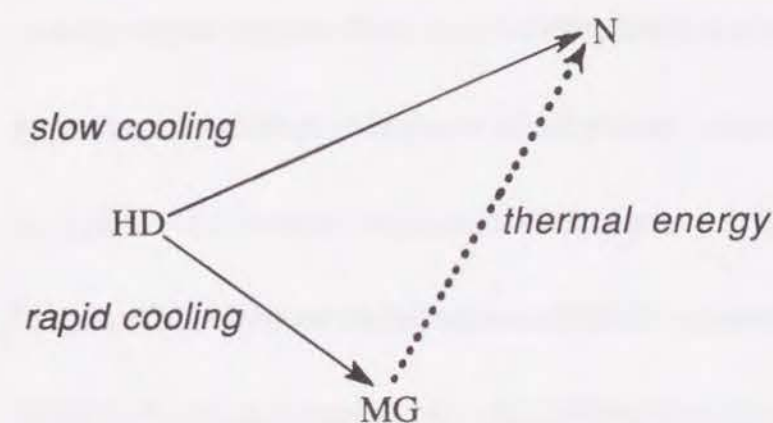
Does the formation of kinetically trapped intermediates depend on the initial state of denatured ovalbumin, namely, the heat-denatured state or the chemically unfolded state? Ovalbumin with a stable but altered conformation, which is richer in hydrophobic regions than the native conformation, was produced from a chemically denatured form of the protein (Klausner *et al.*, 1983). Takahashi and Hirose (1992) showed that the disulfide-bond ovalbumin cannot acquire resistance to trypsin immediately after its refolding under oxidative conditions and, moreover, that trypsin-sensitive species slowly changed into protease-resistant species. It is conceivable, therefore, that the kinetically trapped intermediate is a

general feature of the folding of ovalbumin, regardless of the initial conformation of the denatured protein, although the populations of non-native species produced are expected to be different in each case (compare Figures 10B and 10F).

Implications for protein folding

Partitioning between alternative pathways

The experimental results given in the present paper indicated unanimously that heat-denatured ovalbumin (HD) can either refold to yield the native state (N) during slow cooling or it can condense to a molten globule state (MG) upon rapid cooling, and then the rapidly cooled species changes slowly but spontaneously to the native molecule. Thus, the author can depict the kinetic partitioning between alternative folding pathways, according to a given set of circumstances, as shown in Scheme 1.



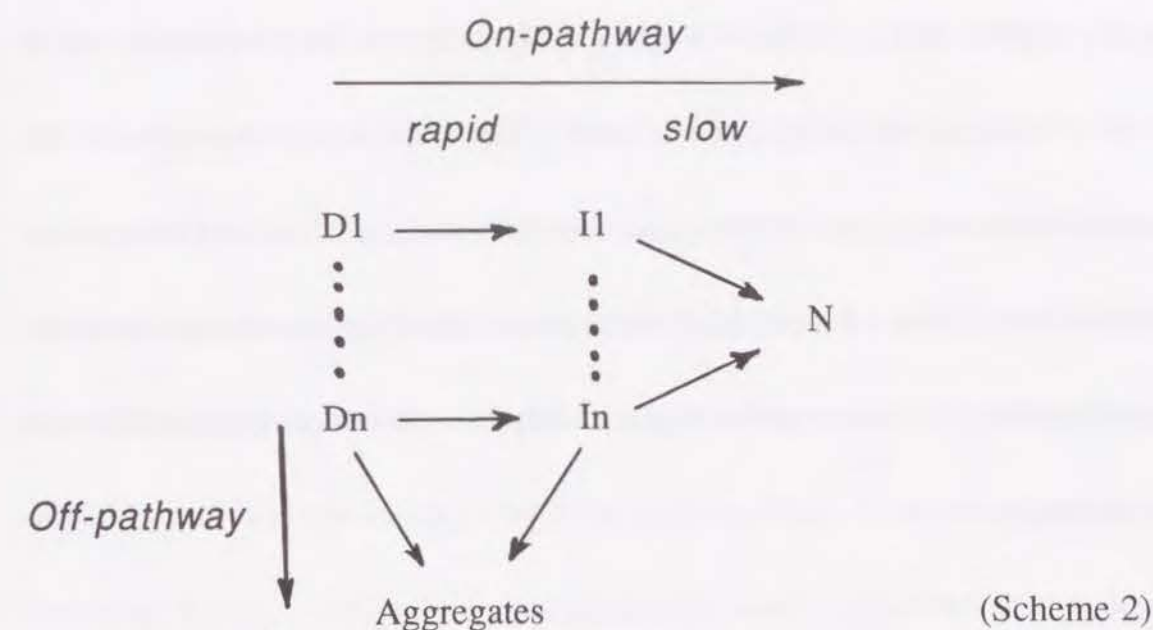
(Scheme 1)

Hydrophobic collapse and slow rearrangement

In our temperature-jump experiment, the yield of renatured protein increased with decreases in the folding temperature from 80°C to 55°C, while the yield decreased at folding temperatures below 55°C (Figure 13). As the folding temperature was lowered, the amplitude of the rapid phase predominated in the total change in expected ellipticity at 222 nm (Figure 13A) and the folding rate during the slow phase changed in the opposite manner (Figure 13B). The reciprocal correlation between the rapid-phase amplitude and the folding rate suggests that the rapid phase represents the conversion of a thermally denatured molecule to an intermediate with molten globule characteristics. The subsequent slow phase is considered to reflect the conformational rearrangement of an intermediate to yield the native protein.

In the rapidly-cooled product, namely, the intermediate with molten globule features, the tryptophan residues are accommodated in a hydrophobic environment. The protein is condensed and globular with a Stokes radius that is close to that of the native protein. In addition, the intermediate accumulates as a variety of disulfide isomers. As shown in Scheme 2, different populations of heat-denatured ovalbumin molecules (Dn) collapse hydrophobically via diverse routes in a rapid phase, and the author has confirmed that an initial "burst-phase" condensation leads

to some misorganization of the polypeptide, with resultant formation of major barriers to folding (Creighton, 1994; Dobson *et al.*, 1994).



At a late stage, the compactly trapped intermediate (In) must expand for the organization of its tertiary structure before it can achieve the native conformation (N). Addition of thermal energy disrupts hydrophobic interactions and breaks a non-native disulfide bond to allow correct pairing with the rearrangement of tertiary topology. Various intermediate molecules cross energy barriers to converge on a native state in the slow phase.

The features of biphasic folding are consistent with a plausible model of protein folding in which the unfolded polypeptide chain undergoes rapid hydrophobic collapse under refolding conditions (Dill, 1985; Camacho & Thirumalai, 1993; Chan & Dill, 1994), perhaps to a molecule that approximates the

molten globule state, with subsequent slow rearrangement to the native form. The evidence supports the proposal of Gregoret and Cohen (1991) that constraints that force the polypeptide chain compact might greatly increase the probability of the final folded conformation being encountered. The evidence also supports the suggestion of Robson and Pain (1971) that the early stage of kinetic folding of a protein should be the formation of a globular molecule upon condensation of the unfolded polypeptide, if we assume that globularity of a native protein stabilizes its secondary structure.

The energy landscape

Folding kinetics can be described in terms of "energy landscapes" concept advocated on the basis of statistical mechanical theories and Monte Carlo simulations of protein folding (Camacho & Thirumalai, 1993; Sali *et al.*, 1994; Bryngelson *et al.*, 1995; Dill *et al.*, 1995; Onuchic *et al.*, 1995; Wolynes *et al.*, 1995). A rugged energy landscape can illustrate the folding pathway of heat-denatured ovalbumin. A slow cooling process induces heat-denatured ovalbumin to refold slowly to the native state. If heat-denatured molecules are cooled slowly enough to allow them to reach thermodynamic equilibrium, a native-like backbone topology can be adopted concomitantly with the rearrangement of incorrect

disulfide pairing. Then the heat-denatured ovalbumin can search for a unique folded conformation with reduction or avoidance of the energy barriers that might prevent structural rearrangements. The behavior of the molecules resembles that of a ball that converges slowly toward the global energy minimum along the surface of the landscape which becomes gradually rugged with slow cooling. By contrast, rapid cooling forces ovalbumin to fold in a kinetically achievable manner. In this process, a flat landscape for denaturing condition changes rapidly to a rugged landscape for folding condition that has many local energy minima and high barriers. A large portion of the molecules in a vast conformational space is trapped as molten globule intermediates in the kinetically accessible states at local energy minima. The existence of several metastable states during kinetic folding of a protein was previously suggested by molecular dynamic simulation of a model β -barrel protein (Honeycutt & Thirumalai, 1990, 1992).

Concluding remarks

Folding of a medium-sized globular protein, ovalbumin, was examined under non-reducing conditions at neutral pH, at a protein concentration and an ionic strength low enough to retard its spontaneous polymerization. Heat-denatured ovalbumin was cooled either slowly or rapidly. Slow cooling allowed the heat-

denatured ovalbumin to refold to its native state, which fact indicated that heat denaturation was reversed by slow cooling. During rapid cooling, by contrast, the heat-denatured molecule collapsed rapidly to an intermediate state that consisted of a variety of compact disulfide isomers with molten globule characteristics. A temperature-jump experiment showed that manipulation of the folding temperature allowed partitioning of heat-denatured ovalbumin between two fates, the renatured protein and the compactly misfolded intermediate. Since the intermediate was able to fold slowly but spontaneously to the native structure, it appeared to be trapped as metastable molecules in a kinetically accessible state. The kinetic barriers that retard rapid refolding were probably due to incorrect disulfide pairings, for the most part if not entirely. The folding of heat-denatured ovalbumin exhibited biphasic kinetics: a rapid phase of hydrophobic collapse and a slow phase of structural organization. Thus, the correct folding of heat-denatured ovalbumin can be achieved by alternative pathways. According to the environment, a reversible transition occurs under thermodynamic control or the folding is governed by kinetic controls. The folding of medium-sized ovalbumin differs from that of a small protein such as α -lactalbumin, in which the molten globule simplifies the search for correct folding by adopting a native-like backbone topology (Wu *et al.*,

1995). The behavior of ovalbumin conforms to theoretical proposals that are based on statistical mechanics, and it can be described by a rugged energy landscape. Ovalbumin seems likely, therefore, to serve as a useful model for elucidation of kinetic mechanisms of protein folding.

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CHAPTER 2

Linear Polymerization Caused by the Defective Folding of a Non-inhibitory Serpin Ovalbumin

Aggregation retards a correct folding of unfolded protein molecules. Unfolded polypeptides are committed either to refold correctly to the native structure or to aggregate to a dysfunctional product. Small proteins such as apomyoglobin and α -lactalbumin tend predominantly to refold rapidly and spontaneously (Kim *et al.*, 1993). By contrast, large proteins are likely to refold slowly and inefficiently because of their more complicated structures. Moreover, as a polypeptide chain increases in length, the increased susceptibility to genetic mutations makes a large protein vulnerable to a structural organization during its folding process (Dobson *et al.*, 1995).

Mutations of a serine protease inhibitor (serpin) trigger a defective folding of the variants that leads to the polymerization of misfolded proteins, the cause of a diverse range of diseases (Stein & Carrell, 1995). Human α_1 -antitrypsin, an archetypal member of the serpin superfamily, is synthesized in hepatocytes of the

liver (Travis & Salvesen, 1983; Sifers & Woo, 1987) and secreted into the circulation as a component of the plasma (Travis & Salvesen, 1983). Its physiological role is the protection of lung elastin fibers from proteolytic attack by neutrophil elastase (Gadek *et al.*, 1981). Some genetic variants of this inhibitor, for example the Z type variant (Laurell & Eriksson, 1963, Jeppsson, 1976), caused aggregation of the protein in the endoplasmic reticulum of hepatocytes to induce liver damage, plasma deficiency of the inhibitor and ultimately, lung emphysema (Stein & Carrell, 1995; Brantly *et al.* 1988; Crystal, 1989, Sifers *et al.*, 1989). Recently Yu and colleagues demonstrated clearly that, unlike wild type α_1 -antitrypsin in which folds in minutes, the folding of Z type variant of α_1 -antitrypsin is extremely slow (Yu *et al.*, 1995) and a cause of aggregation (Sifers *et al.*, 1995). As the mechanism for spontaneous aggregation of the Z variant, a persuasive model of loop-sheet polymerization has been proposed, in which the mobile reactive-center loop of one molecule is inserted into the central β -sheet of another (Lomas *et al.*, 1992; Sifers *et al.*, 1992; Mast *et al.*, 1992; Lomas *et al.*., 1993).

Ovalbumin, a globular protein of chicken egg white with a molecular size of 45 kDa, is a member of the serpin superfamily along with α_1 -antitrypsin (Huber *et al.*, 1989). Ovalbumin shows no inhibitory activity (Long *et al.*, 1980; Ødum *et al.*,

1987), despite sequence homology of about 30% with antitrypsin, but acts as a substrate of elastase (Wright *et al.*, 1984), and hence is classified as a non-inhibitory serpin. The crystal structure of native ovalbumin (Stein *et al.*, 1991) indicates that a five-stranded β -sheet (β -sheet A) runs parallel to the long axis of the molecule and that an α -helix protrudes as a loop that forms the reactive center (see also Fig. 10A in Discussion). Native ovalbumin has a disulfide bond in a solvent-accessible environment and four cysteine residues with free sulfhydryl groups within the intramolecular hydrophobic core (Stein, P. E.*et al.*, 1991). Considering that ovalbumin has both thiol and disulfide moieties, we can interpret that non-native disulfide pairings are formed owing to a rapid thiol-disulfide exchange under non-reducing and denaturing conditions (Tatsumi *et al.*, 1994), and that they disturb a correct folding of chemically-denatured ovalbumin (Takahashi *et al.*, 1992). Recently it was demonstrated that rapid cooling caused a defective folding of heat-denatured ovalbumin as a result of non-native disulfide pairings, and that the rapidly-cooled ovalbumin assumed a compactly misfolded conformation with molten globule-like characteristics as a folding intermediate (Tani, F. *et al.*, submitted for publication). This compactly misfolded ovalbumin was shown to remain monomeric at a low ionic strength. On the other hand, when heated at 80°C

in the presence of salt, ovalbumin produces a highly-ordered linear polymer (Koseki *et al.*, 1989) as does α_1 -antitrypsin (Stein & Carrell, 1995; Mast *et al.*, 1992). Ovalbumin molecules in a chain polymer were found to adopt molten globule-like structures (Tani, F.*et al.*,1995).

In the present study, the author examined the details of molecular interaction for polymerization of ovalbumin by manipulating the ionic strength of the medium that contains the compactly misfolded protein. The mechanism of linear polymerization of misfolded ovalbumin will be discussed in the light of a defective folding and polymerization of serpins that are associated with diverse diseases.

MATERIALS AND METHODS

Materials

Ovalbumin was purified from the egg white of newly laid hens' eggs by crystallization in a solution of ammonium sulfate and was recrystallized five times (Sørensen & Höyrup, 1915). The monoclonal antibody raised against hen ovalbumin that had been denatured by γ -ray irradiation was produced according to a conventional method by Koseki *et al.* in our laboratory (manuscript in preparation). A sequence-specific Fab fragment was prepared by the cleavage of this monoclonal antibody with papain. For detection of bovine BiP, the monoclonal antibody which recognize the consensus sequence of heat shock protein 70 family (Kurtz, S.*et al.*,1986) was obtained from Affinity BioReagents Inc. (Neshanic Station, NJ, USA). From Organon Teknika Corp. (Durham, NC, USA) were purchased a polyclonal antibody raised against hen ovalbumin and alkaline phosphatase-conjugated goat anti-rat IgG antibody with no cross-reactivity to mouse IgG. Protein A sepharose was purchased from Pharmacia (Uppsala, Sweden). Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Promega (Madison, WI, USA). Papain, trypsin (type III), chymotrypsin (type II), and BSA (Fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *Achromobacter* protease I (EC 3.4.21.50) was obtained from

Wako Pure Chemical Industries (Osaka, Japan). IAEDANS was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Iodoacetoamide and urea of specially prepared reagent grade and other chemicals of guaranteed grade were obtained from Nacalai Tesque (Kyoto, Japan).

Methods

Salt-induced polymerization of misfolded ovalbumin

A solution of ovalbumin at 0.25 mg/ml, containing 0.01 mM EDTA, was adjusted to pH 7.5 with 0.1 N NaOH and a pH meter (Toko Chemical Lab. Co., Ltd., Tokyo, Japan). After adjustment of the pH, an aliquot of the solution was heated at 80°C for 5 min in a boro-silicated glass tube and cooled rapidly in an ice-water bath to obtain the compactly misfolded ovalbumin. The solution of the misfolded ovalbumin was concentrated by membrane filtration (Centriprep-30™; Amicon, Beverly, MA, USA) to a protein concentration above 2 mg/ml. As samples of ovalbumin aggregates for size-exclusion chromatography, the misfolded ovalbumin was incubated at 1 mg/ml at 37°C for various periods (1, 6, and 24 h) in 10 mM Tris (pH 7.5) containing 50 mM KCl. As the samples for TEM observation, the protein solution was incubated at 2.8 mg/ml at 37°C for 5 h in 20 mM Hepes (pH 7.5) containing KCl at 50 mM, 150 mM, and 450 mM, or the buffer without KCl.

Size-exclusion chromatography

The aggregates of misfolded ovalbumin were analyzed on a column of TSKgel G3000SW_{XL} (7.8 i.d. x 300 mm; Tosoh, Tokyo, Japan). The column was equilibrated with 20 mM potassium phosphate buffer (pH 7.5), and the samples were eluted with the same buffer at a flow rate of 0.5 ml/min. The amount of ovalbumin applied onto the column was 2.5 µg.

Precipitation and perpendicular light scattering

For the precipitation experiment, the solution of misfolded ovalbumin was incubated at 1 mg/ml at 37°C in the presence of 10 mM Hepes (pH 7.5) containing different anions. After the incubation for 30 min, the samples were stood in ice-bath water for 5 min, and centrifuged for 10 min at 10,000 x g. The absorbance of the supernatant obtained was measured at 280 nm.

For the light scattering experiment, 20 mM Hepes (pH 7.5) containing a variety of anions at 300 mM was placed in a cuvette that was thermostatically maintained at 37°C. Polymerization was initiated by the addition to the cuvette of equal volumes of the misfolded ovalbumin solution to give a final concentration of 1 mg/ml. Perpendicular light scattering was monitored for 1 h at 320 nm in a fluorometer (F-3000; Hitachi, Tokyo, Japan).

Identification of exposed sulfhydryl groups

A solution of Tris buffer containing IAEDANS was added to an aliquot of ovalbumin solution to give a final concentration of 0.13 mg/ml in 50 mM Tris (pH 8.2), 1 mM EDTA and 10 mM IAEDANS, for labeling of the exposed free sulfhydryl groups. After incubation at 37°C for 15 min, the protein was precipitated with a mixture of cold acetone and 1 N HCl (98:2, v/v), washed three times with a mixture of cold acetone, 1 N HCl and H₂O (98:2:10, v/v), dissolved in buffer [50 mM Tris (pH 8.2), 1 mM EDTA] that contained 9 M urea to give a protein concentration of 0.25 mg/ml, and then fully reduced by incubation with 5 mM DTT at 37°C for 30 min. The reduced protein was mixed with 160 mM iodoacetamide, and alkylation of protein sulfhydryl groups was achieved by incubation at 37°C for 10 min. As a standard sample, ovalbumin was fully reduced and unfolded at 37°C for 30 min in the presence of Tris buffer containing 9 M urea and 5 mM DTT. After the incubation, disulfide-reduced ovalbumin was labeled at 37°C for 15 min with 15 mM IAEDANS. The labeled proteins were precipitated with acetone-HCl, washed as described above, and extensively digested by two-step proteolysis. Procedures for two-step proteolysis and separation of the peptides were those developed by Tatsumi *et al.* (Tatsumi *et al.*, 1994). The percentage of

exposed cysteine residues was estimated by the ratio of areas of the peaks showing fluorescent material in a sample to those of the corresponding peaks in the standard.

Purification of bovine BiP

Fresh bovine liver was homogenized in buffer A [20 mM Tris (pH 7.5), 1 mM EDTA, 0.5 mM PMSF and 0.25 M sucrose] with a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 750 x g and then for 10 min at 7,000 x g. The microsome fraction was isolated from the obtained supernatant by sedimentation for 60 min at 100,000 x g through a 20% sucrose cushion in buffer A. The resultant pellet was collected as the crude microsome fraction and suspended in buffer A. BiP was purified from the crude microsome fraction according to the method by Flynn *et al.* (Flynn *et al.*, 1989). Free ATP as well as ATP bound to bovine BiP purified was removed by precipitation of the protein in saturated (NH₄)₂SO₄ solution containing 10 mM EDTA. For use, the precipitate was dissolved in and dialyzed against 20 mM Tris (pH 7.5).

Assays of polypeptide binding to BiP

For analysis of polypeptide binding, 3.9 µg of purified bovine BiP (50 pmol) was incubated at 37°C for 30 min with 2.3 µg of either the misfolded ovalbumin or the native protein (50 pmol) in a final volume of 20 µl of 20 mM

Tris (pH 7.5) containing 140 mM KCl and 3 mM MgCl₂. The BiP-ovalbumin complexes were separated from free BiP by the addition of protein A sepharose beads (20 µl) that had been preloaded with rabbit polyclonal antibody against hen ovalbumin. After mixing at 37°C for 30 min, the complexes-bound sepharose beads were recovered by centrifugation at 3,000 x g, washed three times with 0.5 ml of the reaction buffer, suspended in SDS-PAGE sample buffer [62.5 mM Tris (pH 7.0), 1% SDS, 10% glycerol, 20 mM 2-mercaptoethanol], and treated in a boiling water bath for 5 min. An aliquot of proteins in the supernatant was electrophoresed on 7.5% SDS-polyacrylamide gel according to the standard method used by Laemmli (Laemmli, 1970) and electroblotted onto a PVDF membrane (Bio-Rad Lab.; Hercules, CA, USA) in the same way as described by Hirano (Hirano, 1989). BiP was probed with rat monoclonal antibody to a peptide containing the sequence conserved in heat shock proteins and the blot was developed with alkaline phosphatase-conjugated goat antibody against rat IgG in a mixture of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

ATPase assays

ATPase activity was measured by the method of Shlomai and Kornberg (Shlomai *et al.*, 1980) using TLC on PEI cellulose (Macherey-Nägel;

Düren, Germany) to separate nucleotides. Purified BiP (1 µg) was incubated at 37°C for 20 min with various concentrations of ovalbumin in a volume of 20 µl of the buffer [20 mM Hepes (pH 7.0), 20 mM KCl, 2mM MgCl₂ and 10 mM (N H₄)₂SO₄] that was supplemented with 8 µM ATP containing 0.5 µCi [α-³²P]ATP. Stimulation of ATPase activity was assayed in the presence of ovalbumin over a concentration range from 10 µM to 0.3 mM. Aliquots of the reaction mixtures (2 µl) were spotted on PEI plates that had been prespotted with 1 µl of a mixture of nonradioactive AMP, ADP, and ATP (each 10 mM). The chromatography was developed up to height of 15 cm in a mixture of 0.5 M LiCl and 1 M formic acid, and then dried. The ATP and ADP spots were located with the aid of an ultraviolet light at 254 nm. The radioactivity corresponding to each spot was counted as the intensity of the pictogram as visualized by a FUJIX Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film Co., Ltd., Tokyo, Japan) and expressed as a fraction of the total recovered in each lane.

Measurement of surface plasmon resonance

In the BIAcore 2000™ system (Pharmacia Biosensor AB, Uppsala, Sweden) (Jönsson *et al.*, 1991), the Fab fragment of the monoclonal antibody raised against radiochemically-denatured ovalbumin [50 µg/ml in 10 mM sodium acetate

buffer (pH 5.0)] was immobilized on the dextran matrix of the sensorchip (CM5) surface with a 1:1 mixture of NHS and EDC. The excess active groups on the dextran matrix were blocked with 1 M ethanolamine (pH 8.5) (Johnsson *et al.*, 1991). The Fab fragment was immobilized at the concentration of 3 ng/mm². The freshly prepared ovalbumin samples were passed over the sensor surface at 25°C at a flow rate of 5 µl/ml at concentrations ranging from 200 to 600 nM. The running buffer used in this experiment was 10 mM Hepes (pH 7.4) containing 150 mM NaCl, 3.4 mM EDTA and 0.005% Tween 20. Kinetic analyses were performed with the supplemental software BIAevaluation 2.0 (Pharmacia). The author calculated the association rate constant, k_{ass} , and dissociation rate constants, k_{diss} , using curve fitting to a simple two-component model of interaction ($A + B = AB$) for a titration of the solution-phase ligand. The equilibrium dissociation constant, K_d , was determined by $k_{\text{diss}}/k_{\text{ass}}$.

Competition experiment

A solution of the misfolded ovalbumin was incubated at 1 mg/ml at 37°C for 18 h with a competitor, either bovine BiP or the anti-ovalbumin Fab fragment, in 20 mM Mops (pH 7.0) buffer containing 150 mM KCl, to allow the formation of ovalbumin polymers, and observed by using a transmission electron microscope

(Hitachi H-700H; Tokyo, Japan). The amount of the competitor was half that of ovalbumin in a molar ratio. Other proteins used for control experiments were BSA and the Fab fragment of the monoclonal antibody raised against transglutaminase, both of which exhibit no reactivity with hen ovalbumin.

TEM techniques

The protein solutions were diluted 15 to 50 fold with the same buffer as the author used for preparing the specimen. The specimen was placed on a carbon-coated electron microscope grid, negatively stained with 2% potassium phosphotungstate, and observed with a transmission electron microscope (Hitachi H-700H; Tokyo, Japan) operating at 100 kV.

RESULTS

Salt-induced polymerization of misfolded ovalbumin

Salt-induced polymerization of the misfolded ovalbumin was examined by size-exclusion chromatography on a column of TSKgel G3000SW_{XL}, which was able to separate proteins up to the molecular size of 5×10^5 kDa with a satisfactory high resolution. In the absence of salt (Fig. 1A), a large portion of the misfolded molecules remained in a monomeric state. By contrast, in the presence of 50 mM KCl, the molecules aggregated easily to form oligomers when incubated for 1 h (Fig. 1B). Prolonged incubation promoted aggregation of the misfolded ovalbumin, as judged from a migration of the peak exhibiting oligomers toward the position corresponding to the void volume (Fig. 1C). A high peak that appeared at the void volume after the incubation for 24 h showed the formation of large polymers of molecular size greater than 10^6 kDa (Fig. 1D). Aggregation became more pronounced as the concentration of salt added increased, and prolonged incubation in the presence of 450 mM KCl gave aggregates too massive to pass through the gel matrix (data not shown). However, it should be noted that the peak remained unaltered at the position of a monomer albeit it decreased in height.

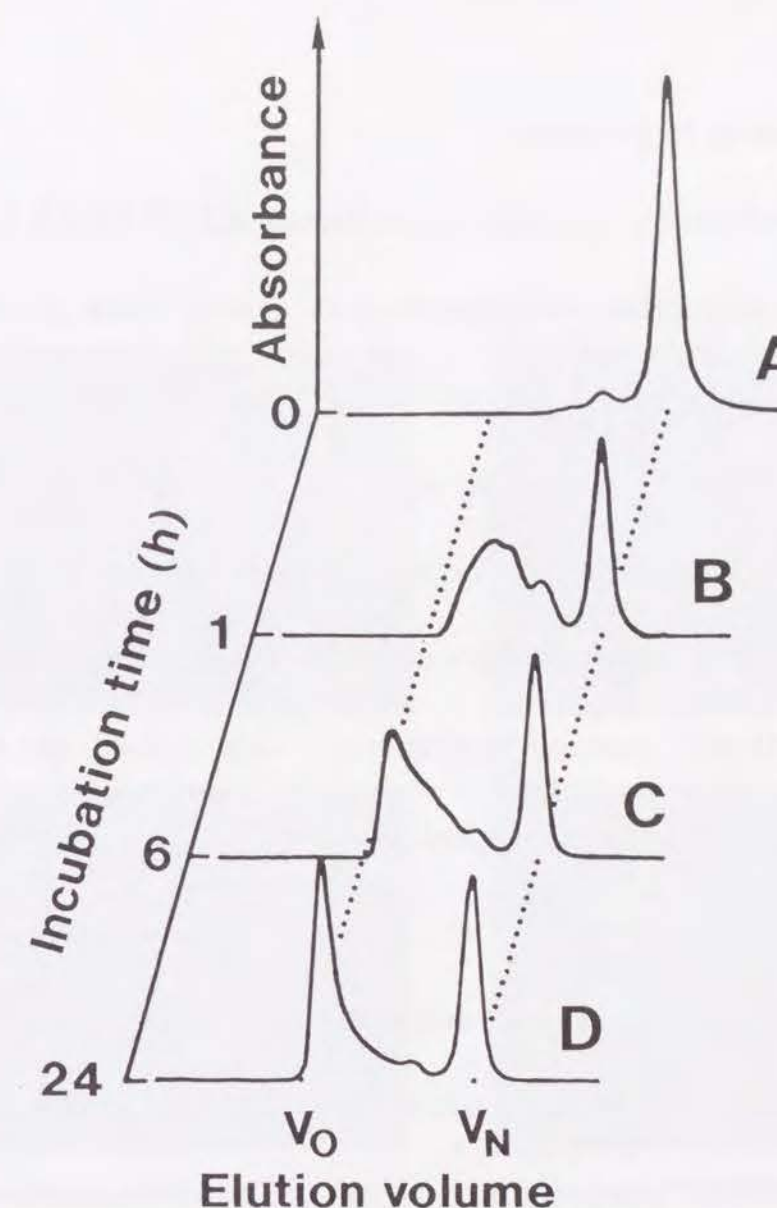


Fig. 1. Comparison of the elution profiles of variously treated samples of ovalbumin during size-exclusion chromatography. Ovalbumin that had been heat-denatured at 80°C for 5 min was cooled rapidly in an ice-water bath. The compactly misfolded ovalbumin was concentrated by membrane filtration (Centriprep-30TM) in the absence of KCl (A). The misfolded ovalbumin solution was incubated at 1 mg/ml at 37°C in the presence of 50 mM KCl for various periods: (B) for 1 h; (C) for 6 h; (D) for 24 h. An aliquot of ovalbumin samples was analyzed on a column of TSKgel G3000SW_{XL} which was equilibrated with 20 mM potassium phosphate buffer (pH 7.5), and the samples were eluted with the same buffer at a flow rate of 0.5 ml/min. The amount of ovalbumin applied onto the column was 2.5 μ g. V_O and V_N indicate the void volume of blue dextran and the elution volume of native ovalbumin, respectively. Absorbance at 280 nm is in arbitrary units.

Shapes of ovalbumin aggregates

When the misfolded ovalbumin was incubated at 37°C for 5 h in the absence of KCl (Fig. 2A), oligomers were observed as a very small particle. In the presence of 50 mM KCl (Fig. 2B), the molecules formed a short, rod-like polymer. The addition of 150 mM KCl allowed the misfolded molecules to assemble in a long linear polymer (Fig. 2C). With the increase in concentration of the salt, linear polymers elongated and some polymers could be observed intertwining. In the presence of 450 mM KCl, massive agglomerates were formed; the formation of ordered fibrous polymers was interrupted and a myriad tangle of filaments was observed (Fig. 2D).

Effect of different anions on polymerization of ovalbumin

Salt with different species of anion induced the misfolded ovalbumin to precipitate at a different concentration of salt. The concentrations of anions required to precipitate the half amount of the misfolded ovalbumin were 0.9 M of sulfate, 1 M of phosphate, and 3.2 M of acetate. Under the conditions the author employed, chloride and iodide appeared to have no effect on precipitating the misfolded protein. However, a high concentration of chloride made the protein solution more viscous than did a similar concentration of iodide. Precipitation of the misfolded ovalbumin was progressively reduced following the order of anions: sulfate, phosphate, acetate, chloride and iodide.

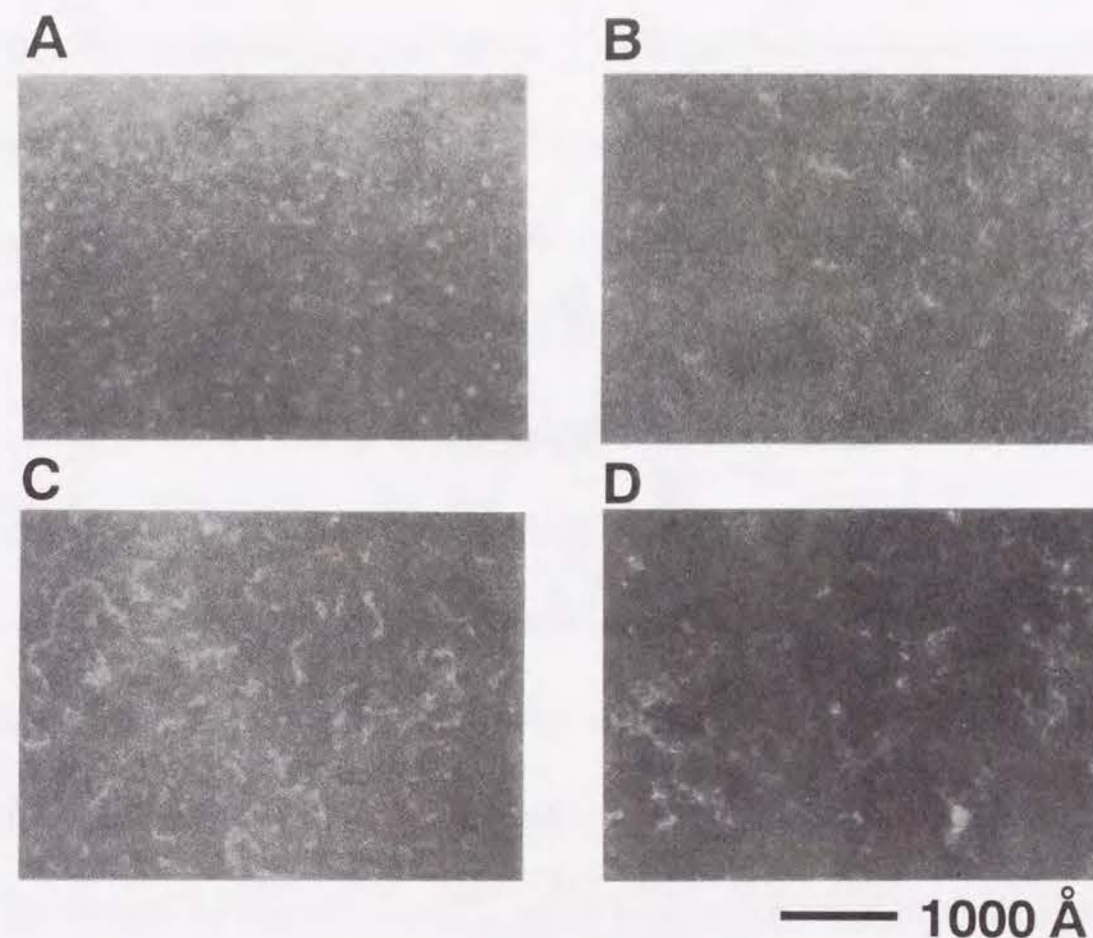


Fig. 2. **Transmission electron micrographs of ovalbumin aggregates.** The compactly misfolded ovalbumin was incubated at 2.8 mg/ml at 37°C for 5 h in the presence of 20 mM Hepes (pH 7.5) containing KCl at 0 mM (A), 50 mM (B), 150 mM (C), and 450 mM (D), respectively. The protein solutions were diluted with the same buffer as the author used for preparing the specimen. The specimen was placed on a carbon-coated electron microscope grid, negatively stained with 2% potassium phosphotungstate, and observed with a transmission electron microscope operating at 100 kV. Resolution was performed at a magnification of x 40 K for photographs.

An increase in light scattering significantly reflects a change of the assembly of macromolecules and in its size and shape. The TEM observation showed linear polymers of the misfolded ovalbumin at the salt concentration of 150 mM, regardless of the type of species of anions. The order of effectiveness of anions in promoting the linear polymerization was shown to be sulfate > phosphate > acetate > fluoride > chloride > bromide > iodide > thiocyanate (Fig. 3).

Identification of exposed sulfhydryl groups

The author examined the behavior of cysteine residues as a consequence of a defective folding of heat-denatured ovalbumin. None of the cysteine residues in the native ovalbumin was labeled with the fluorescent reagent, IAEDANS (data not shown). In contrast, six cysteine residues were all labeled in the misfolded ovalbumin albeit to varying extents (Fig. 4). The percentages of exposed cysteine residues were estimated to be 67.6%, 68.4%, 86.7%, 82.7%, 47.6%, and 59.3% for Cys11, Cys30, Cys73, Cys120, Cys367, and Cys382, respectively. These results suggest that, in the native protein, four cysteine residues (Cys11, Cys30, Cys367 and Cys382) are buried within a hydrophobic core that is sequestered from the solvent molecules. In contrast, the cysteine residues are exposed to a solvent-accessible environment after a rapid cooling of heat-denatured ovalbumin. Another

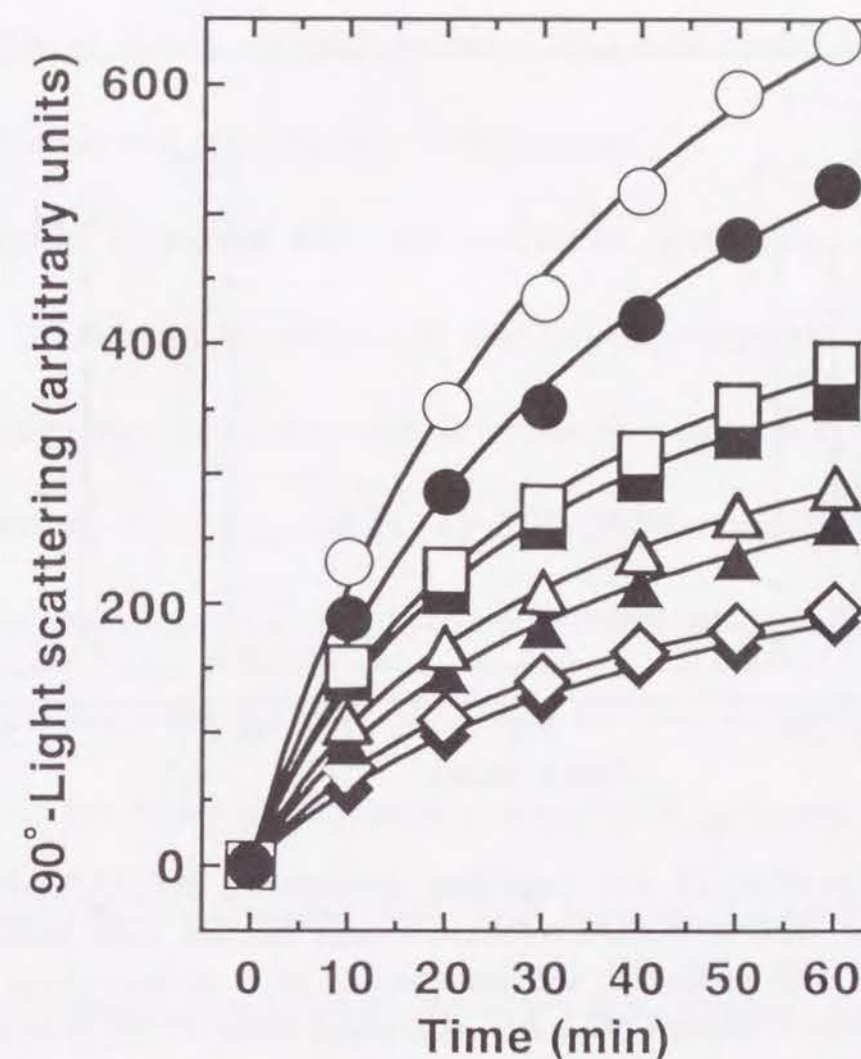


Fig. 3. Effect of different sodium anions on the elongation of chain polymers of ovalbumin in perpendicular light scattering. The misfolded ovalbumin solution was mixed at 37°C with equal volumes of 20 mM Hepes (pH 7.5) containing a variety of anions at 300 mM to give a final concentration of 1 mg/ml. Perpendicular light scattering was monitored for 1 h at 320 nm in a fluorometer. Anions the author used are as follows: sulfate (open circle); phosphate (closed circle); acetate (open square); fluoride (closed square); chloride (open triangle); bromide (closed triangle); iodide (open diamond); thiocyanate (closed diamond).

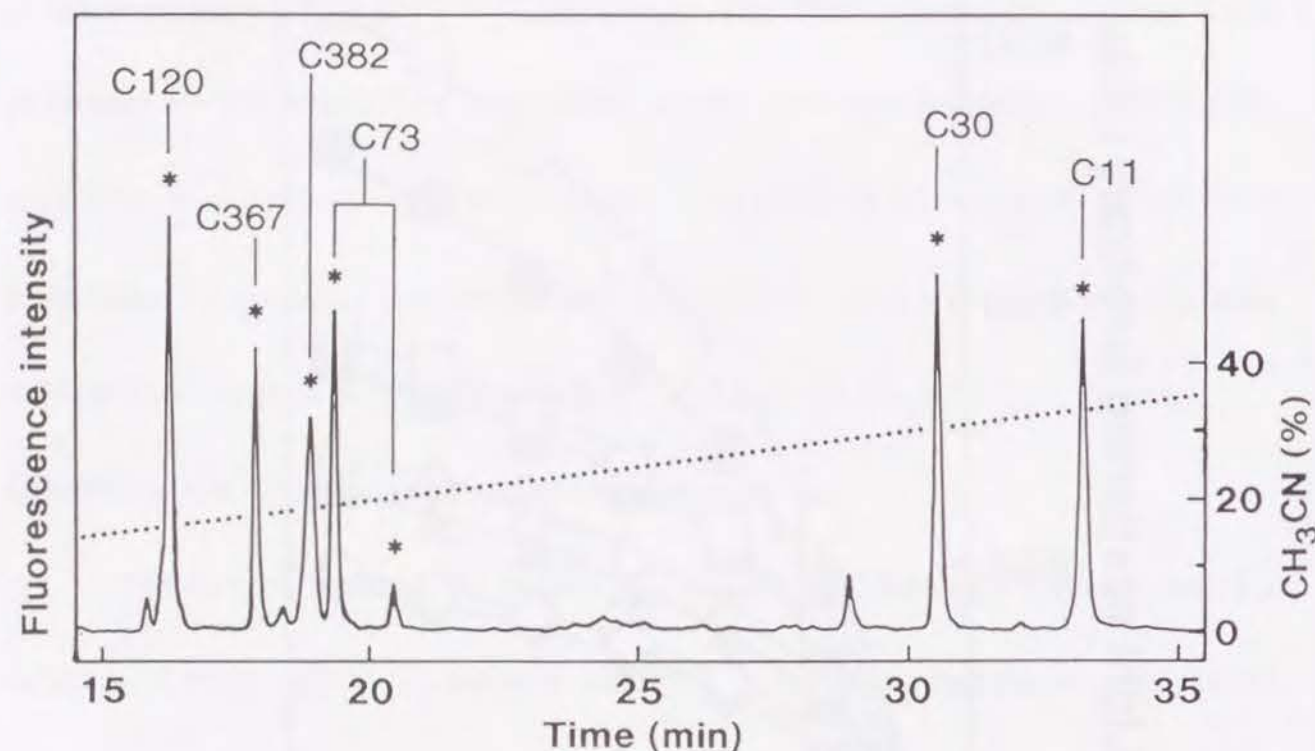


Fig. 4. **Elution profile of the peptides containing AEDANS-labeled cysteine residues.** The misfolded ovalbumin was labeled with IAEDANS, extensively digested with proteases, and fractionated by a reverse-phase HPLC column (4.6 x 150 mm; Cosmosil 5C₁₈-AR) connected to an HPLC system (LC-10AS; Shimadzu) equipped with a spectrofluorometric detector (RF-550; Shimadzu). The seven peaks exhibiting AEDANS fluorescence (excitation, 340 nm; emission, 520 nm), which were identified to be the peptides containing labeled cysteine residues (C), are denoted by asterisks. The numbers following C show the positions of cysteine residues in the primary structure of hen ovalbumin. The percentage of exposed cysteine residues was estimated by the ratio of areas of the peaks showing fluorescent material in a sample to those of the corresponding peaks in the standard.

noteworthy feature was the occurrence of a thiol-disulfide exchange that led to a formation of various disulfide isomers, since both Cys73 and Cys120 that had formed an original disulfide bond were labeled.

Interaction of bovine BiP with misfolded ovalbumin

The two-step procedure with DEAE ion-exchange and ATP-agarose affinity chromatography yielded bovine BiP that was estimated to be 98% pure by densitometry following analysis by SDS-PAGE and staining with Coomassie Brilliant Blue (Fig. 5A). To determine whether purified BiP interacts with the misfolded ovalbumin, the author incubated BiP with ovalbumin samples and then recovered BiP bound to ovalbumin with protein A sepharose beads that had been precoated with anti-ovalbumin polyclonal antibody. Qualitative analysis from the immunoblotting revealed that the recovery was significantly high when BiP reacted with the misfolded ovalbumin compared to the native protein (Fig. 5B). The faint band in the BiP binding to the native protein results from a direct interaction of BiP with antibody-coated sepharose through no mediation of ovalbumin. Therefore, the difference in the intensity between both bands reflects the intrinsic binding of bovine BiP to the misfolded ovalbumin molecules.

Binding specificity of bovine BiP was also confirmed by the stimulation of polypeptide-dependent ATPase activity (Fig. 6A). The native ovalbumin did not

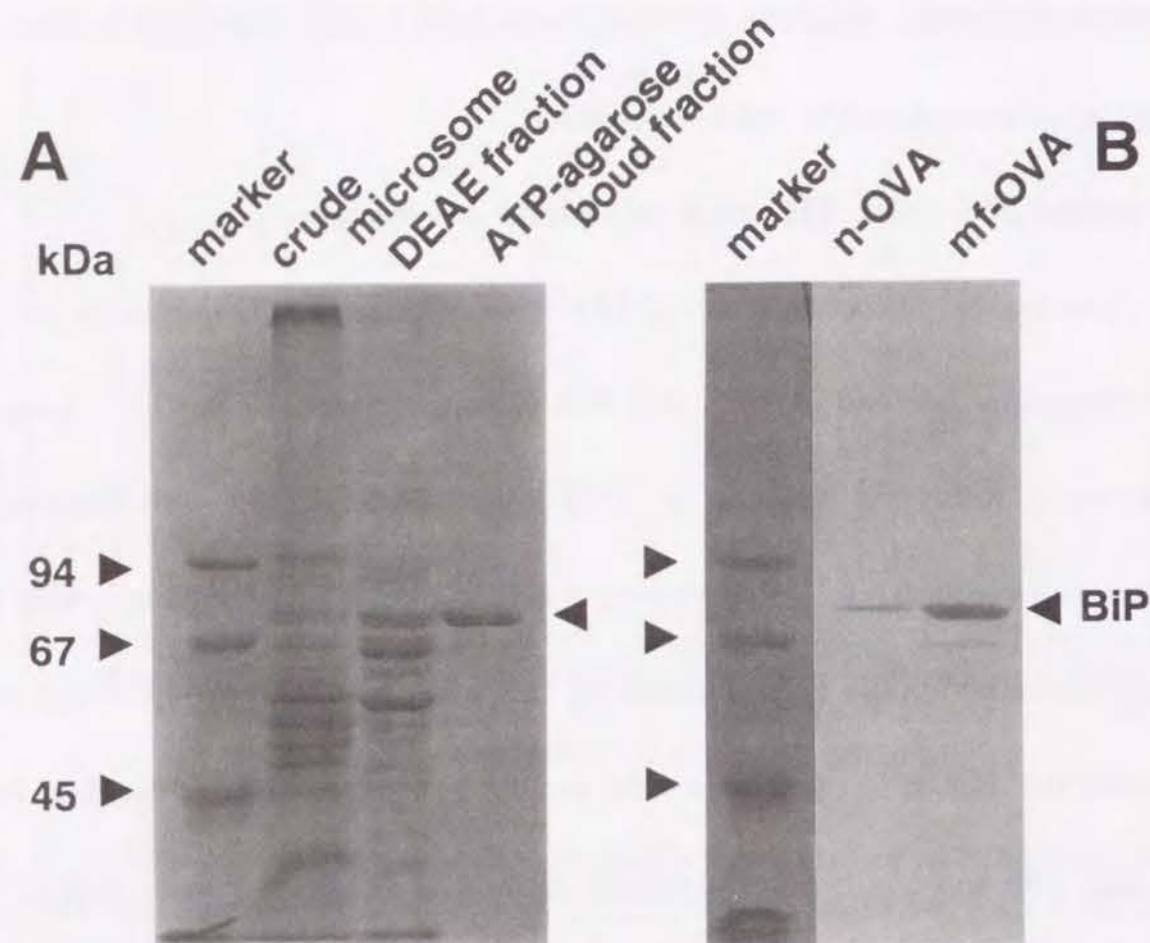


Fig. 5. **Purification of bovine BiP (A) and specific binding of bovine BiP to the misfolded ovalbumin (B).** (A) The collected microsomes were solubilized by Triton X-100 and passed over a DEAE cellulofine column. The BiP-containing fraction was eluted with 200 mM NaCl, and the eluate was loaded onto an ATP-agarose column. BiP was eluted by addition of ATP. A 7.5% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue shows the protein species present in aliquots of samples from different stages of the procedure for purification of bovine BiP. (B) Bovine BiP was incubated with either the native ovalbumin (n-OVA) or the misfolded protein (mf-OVA). The complexes were recovered with protein A sepharose beads that had been precoated with anti-ovalbumin antibodies, analyzed by SDS-PAGE, and then transferred to a PVDF membrane. BiP was probed with rat monoclonal antibody to a peptide containing a heat shock conserved sequence.

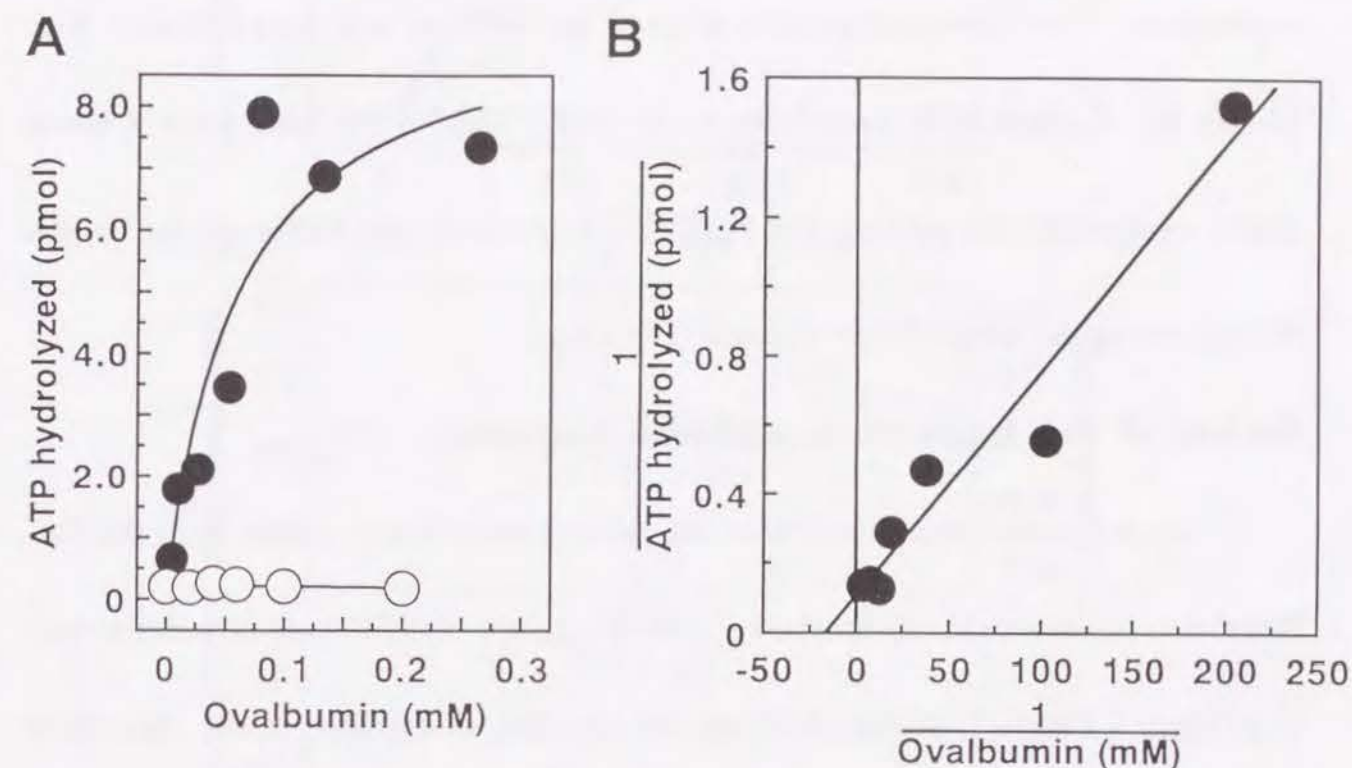


Fig. 6. **ATPase activity of bovine BiP stimulated by the misfolded ovalbumin.** Measurements of ATPase activity were done as described in "MATERIALS AND METHODS". (A) ATP hydrolysis by bovine BiP is a function of the concentration of the misfolded ovalbumin (closed circles) and the native protein (open circles). (B) Double reciprocal plot for the misfolded ovalbumin gives the kinetic parameters of the K_m of 64 μ M and the V_{max} of 0.5 nmol/min per milligram for ATPase activity of bovine BiP.

display any ATPase stimulatory activity of bovine BiP. In contrast, the initial rate of ATP hydrolysis by BiP was dependent on the concentrations of the misfolded ovalbumin. The misfolded protein elicited the ATPase activity of bovine BiP, having the K_m for ATP hydrolysis of 64 μM . The kinetic data gave a linear double-reciprocal plot yielding the V_{max} of 0.5 nmol/min per milligram for bovine BiP driven by the misfolded ovalbumin (Fig. 6B).

Binding of Fab fragment to misfolded ovalbumin

As a ligand immobilized onto the dextran matrix, the author used the Fab fragment of monoclonal antibody raised against radiochemically-denatured ovalbumin. Panel A in Fig. 7 shows the titration of binding of the misfolded ovalbumin to the immobilized Fab fragment. Injection of the native ovalbumin did not result in a rise in sensorgram. In contrast, a significant rise in sensorgram was observed in a concentration-dependent manner for the misfolded ovalbumin. Curve fitting analysis of the binding phase of these curves gave the estimation of an association rate constant k_{ass} of $2.86 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 7B) and a dissociation rate constant k_{diss} of $1.15 \times 10^{-4} \text{ s}^{-1}$ (Fig. 7C). These calculated values suggested that the misfolded ovalbumin associated slowly with and bound ovalbumin dissociated slowly from the immobilized Fab fragment. The equilibrium dissociation constant

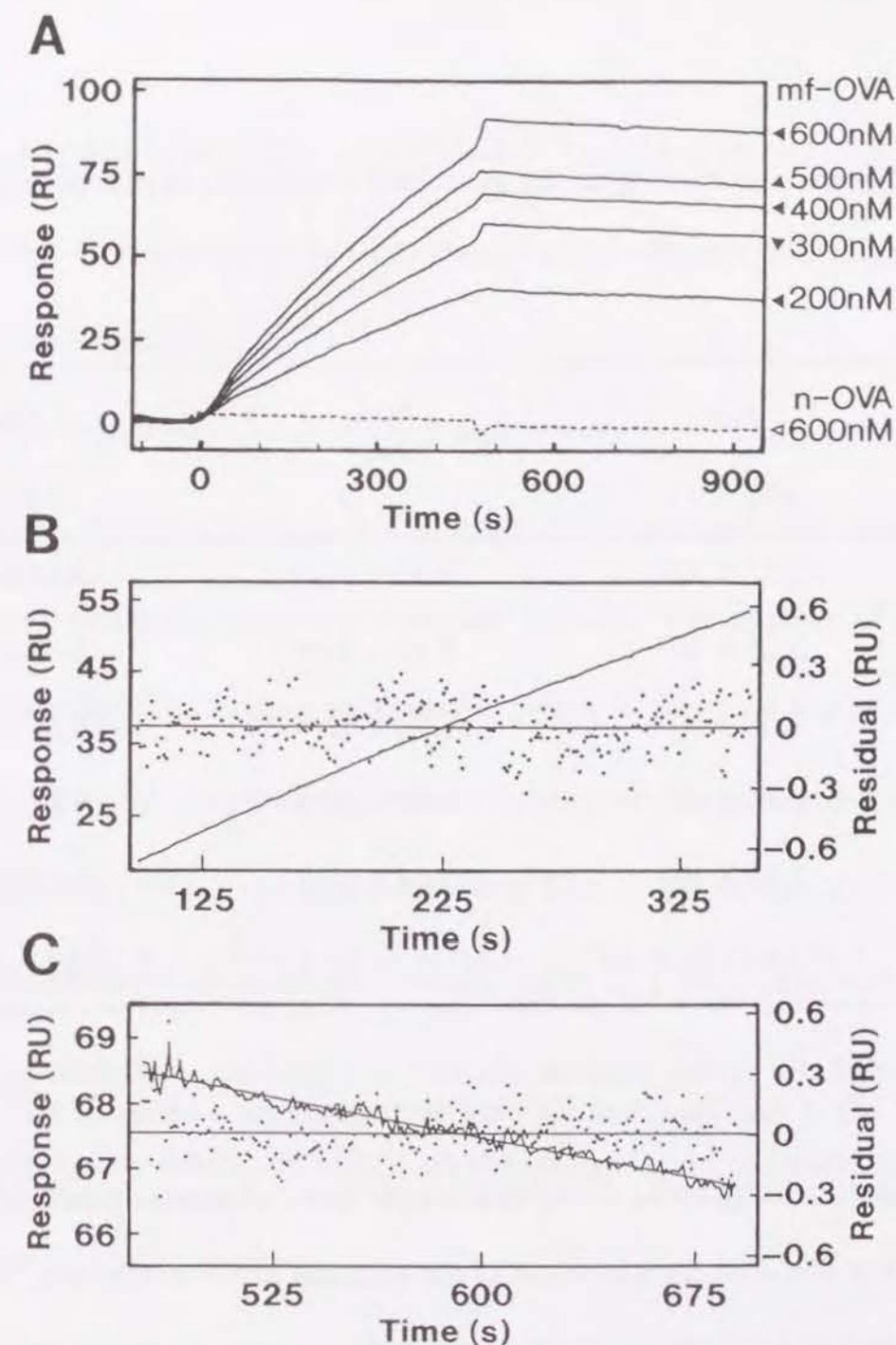


Fig. 7. Surface plasmon resonance measurement of the interaction of the misfolded ovalbumin with the immobilized Fab fragment. The immobilized ligand was the Fab fragment of monoclonal antibody which was raised against radiochemically-denatured ovalbumin. (A) Kinetic analysis of concentration-dependent binding to the immobilized Fab fragment of the misfolded ovalbumin. These curves gave a good fit to a two-component model of interaction. (B) and (C) are representative examples (400 nM) of the curve fitting and the residual plots of the association ($\chi^2 = 0.014$) and dissociation ($\chi^2 = 0.013$) phases, respectively.

Table 1. Kinetics for binding of the misfolded ovalbumin to immobilized Fab fragment

Ovalbumin (nM)	k_{ass} (M ⁻¹ s ⁻¹)	k_{diss} (s ⁻¹)	K_{d} (M)
200	3.55 x 10 ³	1.12 x 10 ⁻⁴	3.15 x 10 ⁻⁸
300	3.15 x 10 ³	1.10 x 10 ⁻⁴	3.49 x 10 ⁻⁸
400	2.68 x 10 ³	1.21 x 10 ⁻⁴	4.51 x 10 ⁻⁸
500	2.65 x 10 ³	1.29 x 10 ⁻⁴	4.87 x 10 ⁻⁸
600	2.26 x 10 ³	1.04 x 10 ⁻⁴	4.60 x 10 ⁻⁸
\bar{X} (\pm s.e.)	2.86 (\pm 0.25) x 10 ³	1.15 (\pm 0.05) x 10 ⁻⁴	4.12 (\pm 0.38) x 10 ⁻⁸

Mean values (\bar{X}) of k_{ass} , k_{diss} , and K_{d} were determined for a series of Fab-ovalbumin samples using curve fitting to a simple two-component model of interaction ($A + B = AB$) for a titration of the solution-phase ovalbumin, as for Fig. 7. Numbers in brackets represent \pm s.e. Analyses used a method with BIAevaluation 2.0 software (Pharmacia). The equilibrium dissociation constant, K_{d} , was calculated from $k_{\text{diss}}/k_{\text{ass}}$.

K_{d} was calculated to be 4.12 x 10⁻⁸ M (Table 1). These results indicated that the Fab fragment bound specifically to the misfolded ovalbumin with high affinity, but not to the native protein.

Inhibition of elongation of ovalbumin polymer The author examined the inhibitory effects of bovine BiP and the anti-ovalbumin Fab fragment on the formation of ovalbumin polymers catalyzed by KCl. The absence of a competitor allowed the misfolded protein to assemble progressively in a linear fashion (Fig. 8A). However, the polymerization was significantly suppressed by addition of bovine BiP (Fig. 8B). The anti-ovalbumin Fab fragment inhibited markedly a linear polymerization of the misfolded molecules (Fig. 8C). The Fab fragment of monoclonal antibody raised against transglutaminase, native ovalbumin and BSA did not display any inhibitory effects on the elongation of the polymer chain (data not shown).

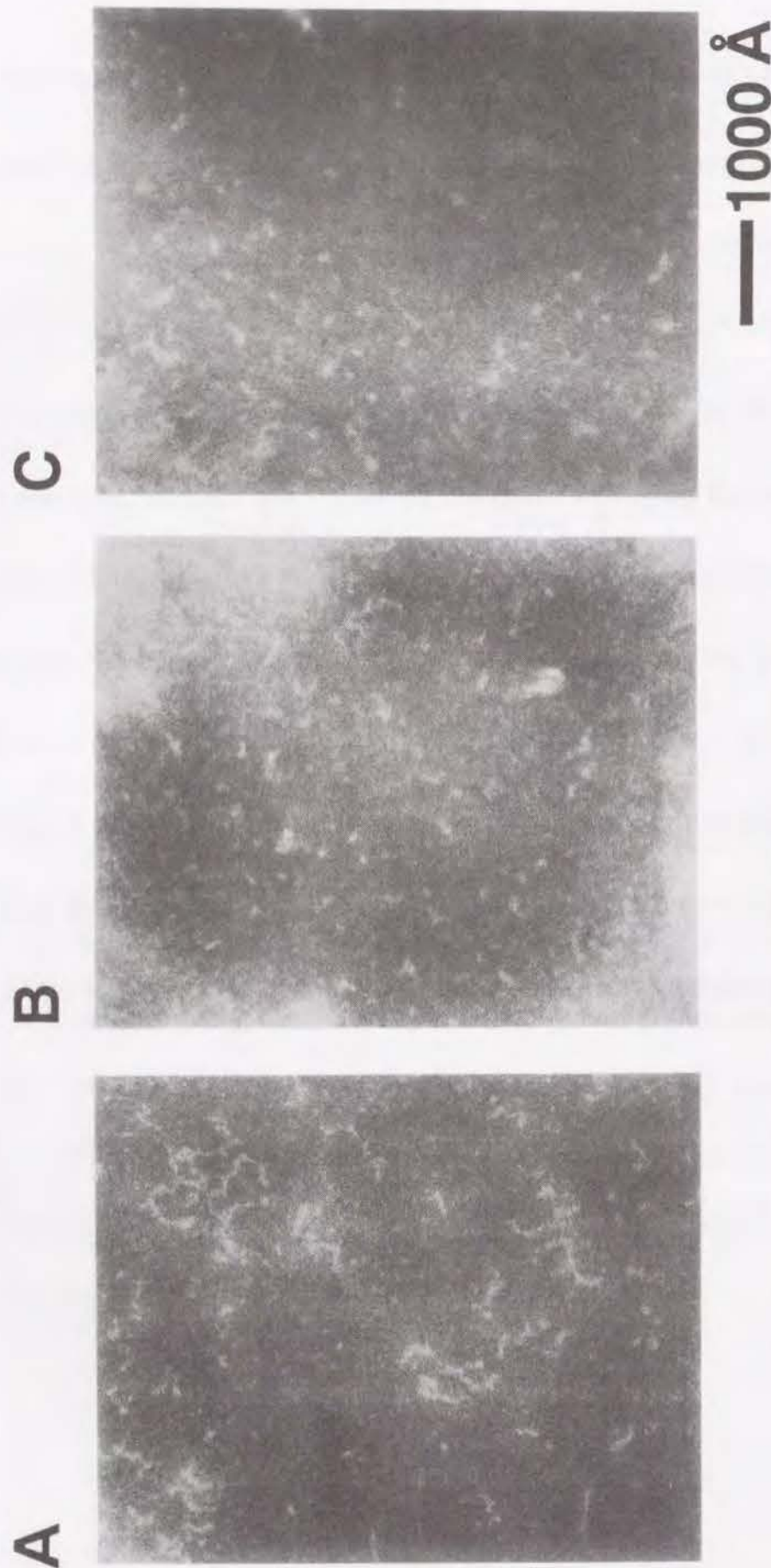


Fig. 8. Transmission electron micrographs showing the inhibition of formation of ovalbumin polymers. Polymerization was conducted in the absence of a competitor (A) and in the presence of either bovine BiP (B) or the Fab fragment of monoclonal antibody raised against radiochemically-denatured ovalbumin (C). Resolution was performed at a magnification of $\times 40$ K for photographs.

DISCUSSION

Results of size-exclusion chromatography (Fig. 1) suggested that the misfolded ovalbumin, which was obtained by cooling heat-denatured protein rapidly, partitions between two fates, non-specific aggregation and productive renaturation. The partitioning between alternative fates depends critically on the concentration of salt added. As salt concentration increased, aggregation developed progressively such that the geometry of the assembly of misfolded ovalbumin changed from a linear polymer into a massive agglomerate (Fig. 2).

The polymerization depended preferentially on the type of species of anions the author employed. The order of effectiveness of anions in promoting the linear polymerization was shown to be sulfate > phosphate > acetate > fluoride > chloride > bromide > iodide > thiocyanate (Fig. 3). This order follows the lyotropic series first demonstrated by Hofmeister for the salting-out of euglobulins (Hofmeister, 1988). Protein conformation can be perturbed by the addition of salts which influence the electrostatic interaction with the charged groups and polar groups, and affect hydrophobic interaction via modification of the water structure (von Hippel & Schleich, 1969; Hatefi & Hanstein, 1969). The degree to which water structure is affected depends on the nature of the anions and follows the lyotropic series (von Hippel & Wong, 1965). Stabilizing anions such as sulfate and phosphate

increase the surface tension of water and cause preferential hydration of a protein, which leads to a more favorable energetic state by the sequestering of nonpolar groups from solvent molecules, thereby enhancing hydrophobic interaction. In contrast, destabilizing anions such as iodide and thiocyanate could reduce the energy required to transfer the nonpolar groups into water, thereby weakening hydrophobic interaction (Hippel & Schleich, 1969; von Hippel & Wong, 1965). Therefore, the preferential effect of anions in the linear polymerization supports a view that denatured protein molecules aggregate through hydrophobic interaction.

Attention should be paid to the interpretation of the preferential effect of anions on linear polymerization (Leberman, 1991), because Hofmeister series pertains to salts at high concentrations. The salt concentration required for linear polymerization of the misfolded ovalbumin could be evaluated as a marginal concentration to exhibit the preferential effect of anions on protein assembly. The effect of anions may be amplified as a result of more increased hydrophobicity in the misfolded ovalbumin than in the native protein. Indeed, when the surface hydrophobicity was compared between the misfolded ovalbumin and the native protein, the former exhibited significant fluorescence of a chromophore, ANS, whereas the latter did not display any fluorescence (Tani, F. *et al.*, submitted for publication). Moreover, the misfolded ovalbumin exposed the cysteine residues in

the hydrophobic sequences that located normally in the interior of the native protein (Figs. 4 and 9).

The involvement of hydrophobic interaction in linear polymerization was confirmed by the finding that the linear polymerization was inhibited by intrinsic binding of bovine BiP to the misfolded ovalbumin (Fig. 8B). Mammalian BiP was shown to have the ability to bind to a variety of peptide sequences with a broad spectrum in hydrophobicity in an ATP-dependent manner (Flynn *et al.*, 1989; 1991; Blond-Elguindi *et al.*, 1993). The K_m of various synthetic peptides for ATPase activity of bovine BiP varied over the ranges from 10 μ M to > 1 mM (Flynn, G. C. *et al.*, 1989; Blond-Elguindi, S. *et al.* 1993). Flynn *et al.* determined the V_{max} of bovine BiP driven by the peptides to be approximately 1.0 to 2.0 nmol/min per milligram (Flynn *et al.*, 1989). Bovine BiP bound to the misfolded ovalbumin but not to the native protein in an ATP-dependent manner (Figs. 5 and 6). The K_m and V_{max} values the author estimated were close in magnitude to those reported before. This specific binding reflects a typical characteristic of the interaction of molecular chaperone BiP with a denatured protein, although the equilibrium dissociation constant remains to be determined.

It thus follows that the compactly misfolded ovalbumin polymerized through hydrophobic interaction occurring among the areas exposed as result of the defective folding of heat-denatured protein.

A sequence-specific Fab fragment of the monoclonal antibody, which was raised against radiochemically-denatured ovalbumin, enabled us to probe a possible interaction site among the misfolded molecules owing to the high specificity of its complementarity determining region. The epitopes that are exposed on a denatured protein but occluded in its polymer are possible interaction sites. As a candidate of interaction sites, therefore, the epitope of sequence-specific antibody that retards the linear polymerization of misfolded ovalbumin molecules can be identified. The Fab fragment the author employed was highly conformation-sensitive with specific binding to misfolded ovalbumin but with no reactivity to the native protein (Fig. 7), and it had a low dissociation constant K_d of 4.12×10^{-8} M to the misfolded protein (Table 1). Since the Fab fragment, the epitope include a sequence of 172 to 183 residues [$^{172}\text{MVLVNAIVFKGL}^{183}$] in the primary structure of hen ovalbumin (Fig. 9) inhibited the linear polymerization of the misfolded ovalbumin (Fig. 8C). The simplest interpretation of our result is, at present, that the sequence of 172 to 183 residues is part of, or close to, one of the regions of axial contact among the

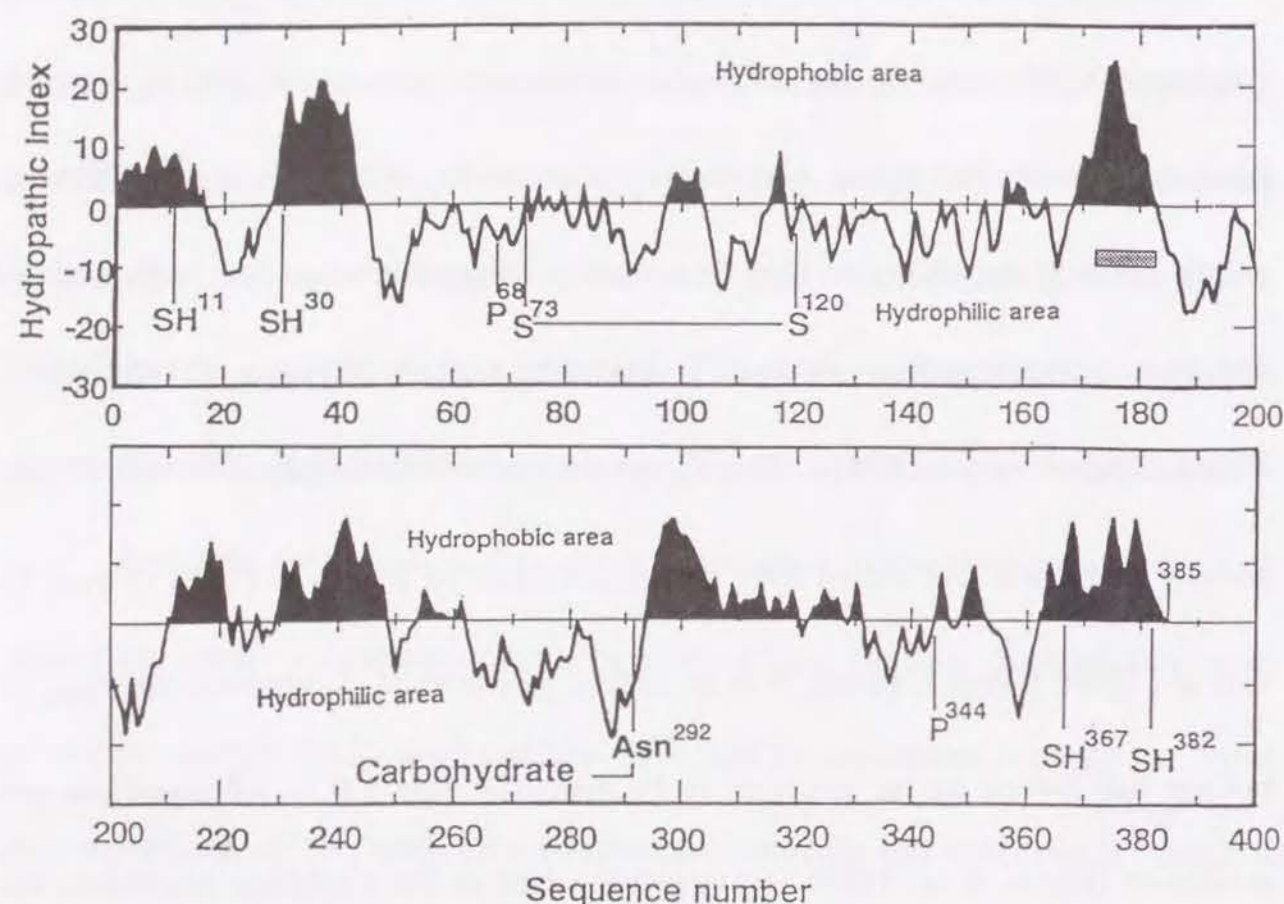


Fig. 9. **Hydropathy profile of the primary structure of native ovalbumin.** The hydropathy profile of ovalbumin was depicted by plotting the averaged hydropathy index (46) of a nonapeptide composed of amino acid residues $i-4$ to $i+4$ versus i , the residue number of the amino acid. The computer program of SDC-GENETYX (Software Developing, Tokyo, Japan) was used for this analysis. The primary structure of ovalbumin reported by Nisbet *et al.* (47) was employed. Hydrophobic regions are drawn black and the epitope of the monoclonal Fab fragment the author employed is delineated by the dotted bar. Free sulfhydryl groups of cysteine residues in the native protein are represented as SH with numbers that show their positions in the primary structure. An original disulfide bond (S-S) is bridged between Cys73 and Cys120.

misfolded ovalbumin. Many aliphatic amino acid residues in the epitope sequence gives evidence in favor of hydrophobic interaction for the polymerization. However, the author cannot exclude the possibility of other segments as interaction sites because the hydropathy index of hen ovalbumin presents seven hydrophobic sequences in the primary structure (Fig. 9).

The mechanism of polymerization caused by a defective folding of heat-denatured ovalbumin can be explained by reference to the model of loop-sheet polymerization of the Z type variant of α_1 -antitrypsin. It seems likely that the molecular structure of the active serpin α_1 -antitrypsin corresponds to that of the native ovalbumin. In the α_1 -antitrypsin cleaved at the reactive-center loop, the amino terminal segment of the loop is incorporated in the gap between β -strands 3A and 5A (Loebermann *et al.*, 1984). The separate peptide corresponding to the incorporated segment prevented the Z variant from spontaneous polymerization (Lomas *et al.*, 1992; Mast *et al.*, 1992). In the proposed model, in which the mobile reactive-center loop of one molecule is inserted into the central β -sheet A of another, an opening of β -sheet A is required for the insertion of the loop. It is noteworthy that the peptide segment of 172 to 183 residues recognized by the Fab fragment is a part of the central β -strand 3A of hen ovalbumin (Fig. 10A). The β -

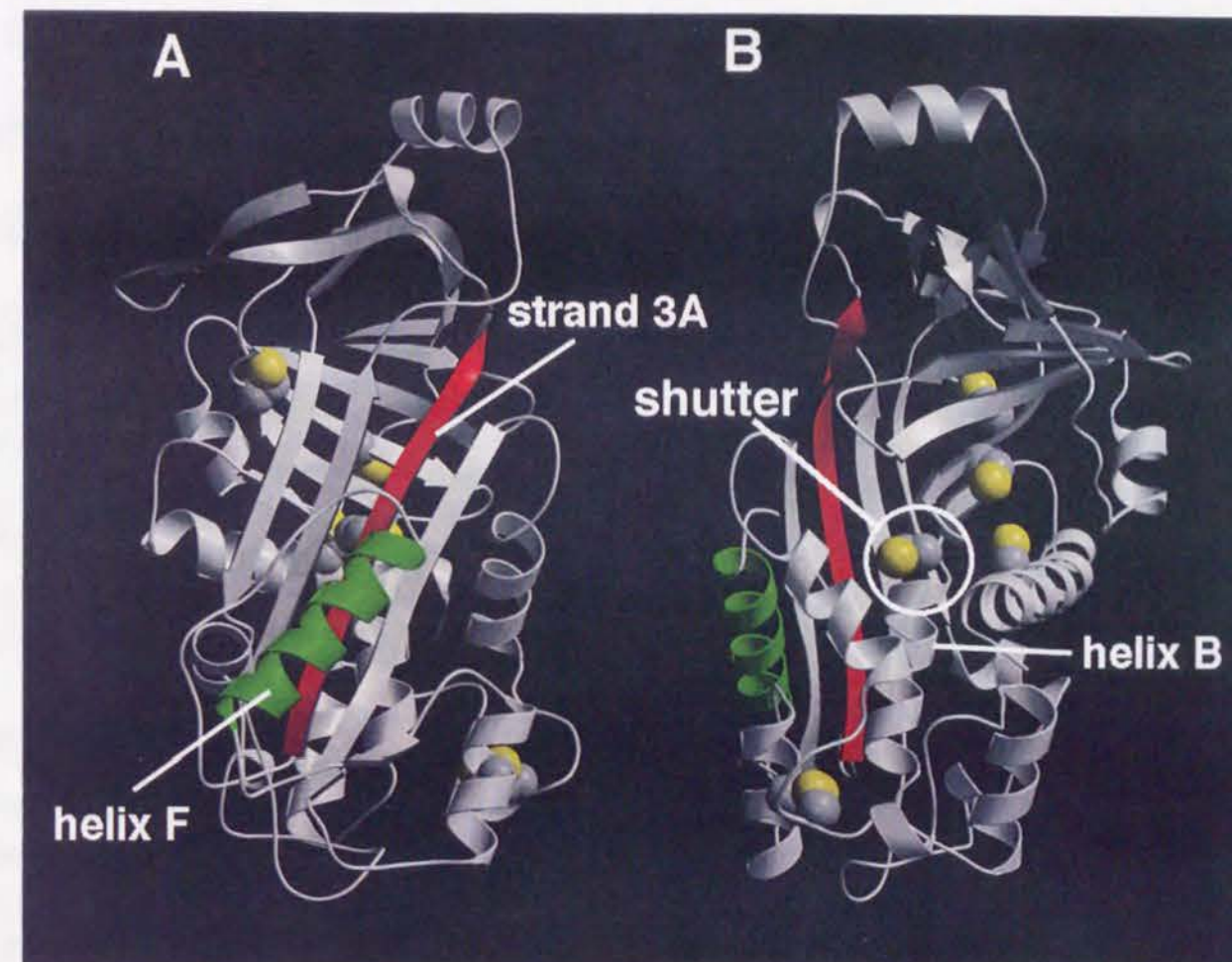


Fig. 10. Schematic representation of the structure of native ovalbumin (23). α -Helices are represented by ribbons and strands of β -sheets by arrows. Sulfur atoms, and both α - and β -carbon atoms in cysteine residues are shown as yellow and gray spheres, respectively. (A) The β -strand 3A containing the epitope of the Fab fragment is represented by red and α -helix F is represented by green. The epitope is packed intramolecularly under the cover of helix F in the native ovalbumin. (B) The shutter domain beneath the β -strand 3A is circled. The domain locates beneath the β -strand 3A and corresponds to the Tyr-Cys-Pro [29-31] sequence at the commencement of helix B in the native ovalbumin. The diagram was drawn using the programs MOLSCRIPT (48) and Raster3D Ver. 2.0 (49).

strand 3A is packed intramolecularly under the cover of helix F in the native ovalbumin. The accessibility of the Fab fragment to the misfolded ovalbumin (Fig. 7) indicates clearly that the structure of, or around, β -sheet A was poorly constructed with the exposure of β -strand 3A during the refolding process of heat-denatured ovalbumin. Evidence for a non-native structure of β -sheet A was circumstantially provided by the labeling of Cys30 (Fig. 4). The 'shutter domain' (Fig. 10B) is known as a functional domain for maintenance of the correct architecture of β -sheet A (Stein & Carrell, 1995). Perturbation of the shutter domain facilitates the polymerization of two α_1 -antitrypsin variants (Frazier *et al.*, 1989; Seyama *et al.*, 1991). This domain locates beneath the β -strand 3A and corresponds to the Tyr-Cys-Pro [29-31] sequence at the commencement of helix B in the native ovalbumin. The labeling of Cys30 in the misfolded ovalbumin indicates a non-native structure of the shutter domain and ultimately an insufficient organization of the central β -sheet A. Therefore, an exposure of the region of, or adjacent to, β -strand 3A in the central sheet A is required for hydrophobic contact among the misfolded ovalbumin molecules.

Analysis of disulfide pairing revealed that the misfolded ovalbumin molecules were composed of a variety of disulfide isomers (Tani, F. *et al.*,

submitted for publication). For this reason, the problem still persists whether the polymerization can be mediated by either a few definite and sequence-specific regions or multiple and non-specific hydrophobic regions. However, it seems likely that disulfide isomers resemble each other in that a large portion of the misfolded molecules formed dimers with the Fab fragment, regardless of differences at local structures. Therefore, we can interpret that a common feature in the defective folding of heat-denatured ovalbumin is exposure of the hydrophobic region of β -sheet A.

In conclusion, an important corollary is that a rapid folding of heat-denatured ovalbumin caused a lack of organization of, or adjacent to, the central β -sheet A, thereby leading to the exposure of hydrophobic regions in the compactly misfolded ovalbumin. The preferential effect of anions and the inhibitory effect of BiP indicated that the compactly misfolded ovalbumin polymerized through hydrophobic interaction. A sequence-specific antibody revealed that a possible interaction site for axial contact among the misfolded molecules was a partial segment of β -strand 3A. This polymerization process may be explained by reference to the mechanism postulated for loop-sheet polymerization as was observed in the Z type variant of a serpin α_1 -antitrypsin. Together with some

genetic variants of serpins, ovalbumin, a non-inhibitory serpin, seems likely to serve as a promising model in elucidating the kinetic mechanism of serpin folding.

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CHAPTER 3

Phosphoryl and Carbohydrate Moieties in Ovalbumin have a Chaperone-like Role of Preventing Aggregation

There are some reports on the effects of carbohydrate moiety on aggregation that course serious problem in vivo., though, few is on the effect of phosphoryl group. For example, deglycosylated Tamm-Horsfall glycoprotein (THP) could not form coaggregation,that causing cast nephropathy, with BenceJones proteins (Huang *et al.*, 1993). And *O*-linked sugar chain of human franulocyte colony-stimulating factor,hG-CSF (Oh-eda *et al.*, 1990), *N*-linked sugar chain of recombinant human erythropoietin, r-EPO (Endo *et al.*, 1992) and genetically introduced sugar chain in egg white lysozyme (Kato *et al.*, 1994) are preventing inactivation accompanied by heat induced aggregation. In these proteins, sugar chains could effect on inhibition of aggregation by next three point; stability of native state, refolding rate and binding affinity between molecules. Except a few case, the cause of inhibitory effect on aggregation was investigated.

On the other hand, some of intracellular proteins, such as heat shock proteins, would related in the inhibition of aggregation. In fact, chaperonin (GroEL) from *Escherichia coli*, that is one of the members of heat shock proteins, has been shown to suppress heat induced aggregation of many proteins (Guagliardi *et al.*, 1995) and to interact mainly to exposed hydrophobic clusters of denatured proteins (Martin & Hartl, 1991; Hoshino & Goto, 1996).

Previous studies on heat induced gelation of OVA suggested that gel networks were formed by hydrophobic interaction within denatured molecules. Since, ovalbumin is a good material to study about the phosphoryl groups and carbohydrate moiety on inhibition of aggregation. In this chapter, the author have prepared dephosphorylated and deglycosylated OVA species enzymatically and investigated the salt induced polymerization of misfolded OVA that caused by heated and rapidly cooled. And then to investigate the existence of hydrophobic clusters on molecular surface, the author compared the binding affinity to GroEL of three species of misfolded OVA in which different in the existence of phosphoryl and carbohydrate moieties.

Materials and Methods

Materials

Dihydrofolate reductase (DHFR, EC 1.5.1.3), casein and acid phosphatase type I from wheat germ 0.8 U/mg (EC 3.1.3.2) were purchased from Sigma and Glycopeptidase F (3.5.1.52) was purchased from Takara(Kyoto,Japan).

Methods

Dephosphorylated ovalbumin species, A1-OVA, was fractionated from purified OVA after acid phosphatase treatment on Q sepharose HP 26/10 (Pharmacia) chromatography and checked the number of phosphoryl groups by polyacrylamide gel electrophoresis (PAGE) as described by Kitabatake in 1988. 1 g OVA was reacted in 25 ml of 250 mM MES-NaOH, pH 5.0, 0.025 mM EDTA, 0.05 mg/ml NaN_3 with 3.1 mg acid phosphatase at 37°C for 52 hours. After adjustment pH 6.0 with 0.1 M NaOH, reacted OVA solution was applied on Q-Sepharose equilibrated with 20 mM MES-NaOH pH 6.0 and eluted with a linear gradient of NaCl (0.5 mM/ml) at 4 ml/min. The numbers of phosphoryl groups was identified with Native-PAGE on 10% gel (Fig. 1A). In order to prepare deglycosylated species from A3-OVA, a part of heat denatured A3-OVA was reacted with

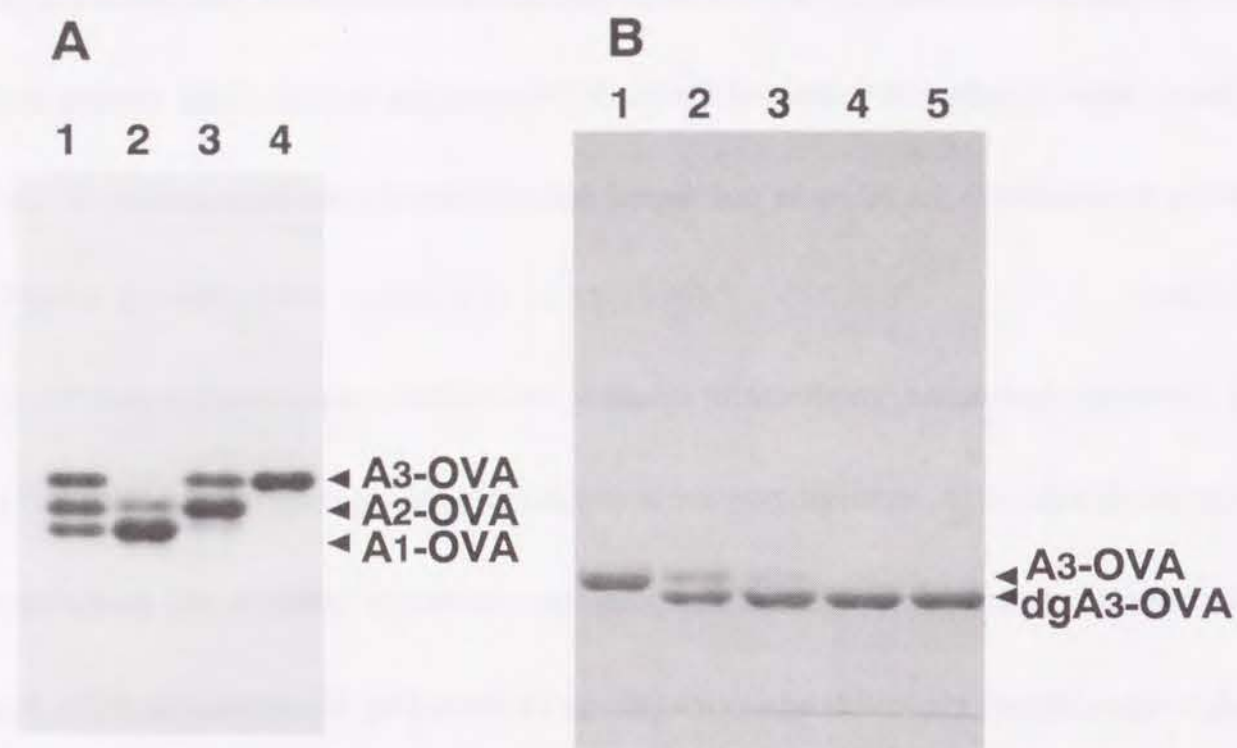


Fig. 1. Preparation of dephosphorylated and deglycosylated ovalbumin species. A1-OVA Formation of ovalbumin species that are resistant to trypsin digestion by alternative cooling processes. (A) Preparation of dephosphorylated OVA, A3-OVA. Lane 1 corresponds to markers. Lane 2 corresponds to OVA purified from hen egg white. Lane 3 corresponds to deglycosylated OVA species in fractions of Q sepharose chromatography. (B) Preparation of deglycosylated OVA, dgA3-OVA. Lane 1 and 2 correspond to A3-OVA before and after Peptide:N-glycosidase F treatment. Deglycosylated OVA was completely purified with Q sepharose chromatography (lane 3), ConA-Agarose column (lane 4) and WGA-Agarose column (lane 5), respectively.

Peptide:N-glycosidase F. 1 mg/ml of A3-OVA in 5 mM Potassium Phosphate, pH 7.5 at 80°C for 15 min, and cooled. Heated OVA solution was concentrated to 8 mg/ml with Centricon-30(Amicon). 60 mg of A3-OVA in 15 ml of 25 mM Tris-HCl, pH 8.6 was reacted with 30 mU of Peptide:N-glycosidase F at 37°C for 40 hours. The deglycosylated OVA species can identified with SDS-PAGE on 12% gel (Fig. 1B). After the reaction 29.7 g urea, 2.75 ml of 1 M Tris-HCl pH 8.2, 275 μ l of 1 M DTT and distilled water were added to give 9 M urea 50 mM Tris-HCl 5 mM DTT and incubate for 30 min. 55 ml of completely unfolded OVA in 9 M urea was diluted into 440 ml of 50 mM Tris-HCl, pH8.2, 1 mM DTT at 20°C and kept for 16 hours. Refolded OVA was concentrated by ultrafiltration with Amicon PM30 (amicon glase) and change the buffer to 50 mM MES-NaOH, pH 6.0. A3-OVA was removed by anion exchange and two lectin columns to remove . First, concentrated OVA solution was applied onto Q-sepharose and eluted in the same condition as described above except flow rate at 2 ml/min. Eluted fraction was checked on 8% SDS-PAGE and then concentrated and changed the buffer with centricon-30 and changed to 50 mM Tris-HCl, pH 7.0, 1 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ containing 0.2 mg/ml NaN_3 . And then applied onto ConA-Agarose

column, (1.8 ml 6-9 x 37 mm, Seikagaku-kougyou) equilibrated with the same buffer. Through fractions were concentrated and changed buffer to 50 mM Sodium Phosphate, pH 7.2 and applied onto WGA-Agarose column (1.8 ml volume, 6-9x37mm, Seikagaku-kougyou) equilibrated with the same buffer.

Measurement of the melting temperature

Samples were dissolved to 0.1 mg/ml in 0.01 mM EDTA and adjust pH value at 8.0. The temperature of the water bath that equipped to CD cell folder was increased at rate of 1 °C/min, monitoring CD $[\theta]_{222\text{ nm}}$ intensity. Real temperature of sample in a quartz cell was measured in the another experiment done in the same condition. And the melting temperature (T_m) of each OVA species in heat denaturation were determined from the next fitting equation with Kaleida Graph version 3.0.5.

$$(c_1t + c_2) + [(c_3t + c_4) - (c_1t + c_2)] / [1 + (T_m / t)]^{c_5}$$

where t is a temperature of sample in centidegree, c_1 to c_5 are the constant decided in fitting, T_m is the transition point in the sigmoidal curve.

CD measurements

The ellipticity at 222 nm or far-UV CD spectra were measured with Jasco

spectropolarimeter, model j-300A, equipped thermal water bath to control the cell temperature. The cell has the length of the light-path of 1 mm. The results were expressed as the mean residue ellipticity, $[\theta]=100 \times q \text{ (lc)}^{-1}$, where q is the ellipticity in degrees, c is concentration in residue moles per liter and l is the length of the light-path in centimeters.

Preparation of misfolded ovalbumin

Ovalbumin was dissolved to 0.1 mg/ml and adjusted pH 8.0 with 0.1 N NaOH was heated at 80°C for 5 min in glass tube (10 x 75 mm) and cool rapidly in ice-water bath. Rapidly cooled solution was concentrated to 1 mg/mg with centricon-30 at 4°C. Trypsin digested samples were checked on 8% SDS-PAGE gel (Fig. 2).

Comparison of the formation rate of salt induced linear polymer of misfolded ovalbumin

Misfolded OVA by heating and rapid cooling were concentrated to 1 mg/ml with Centricon-30 at 4°C. Polymerization was induced by addition of equal volume of 20 mM HEPES-NaOH, pH 7.5, 300 mM Na₂SO₄ in quartz cell at 37°C and light

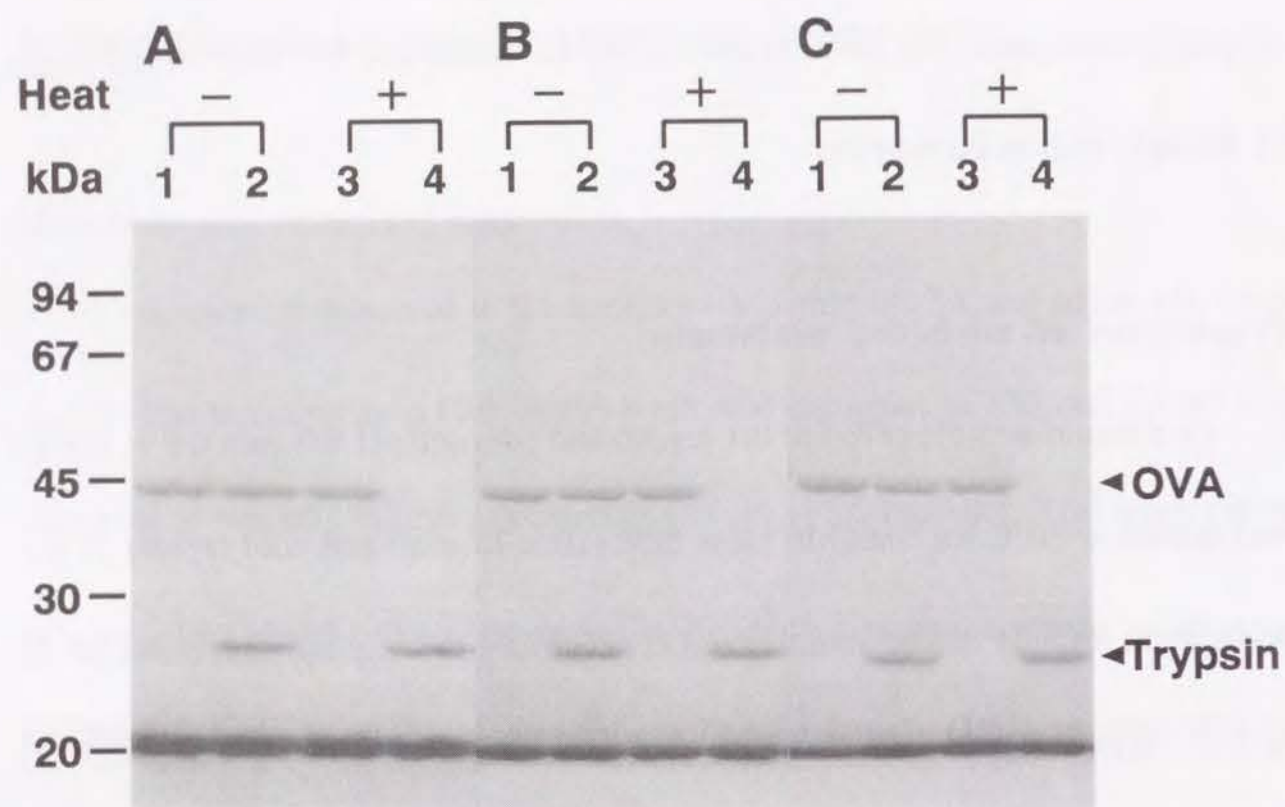


Fig. 2. Trypsin digestion test of before and after heat treatment of three ovalbumin species. (A), (B) and (C) correspond to the A1-OVA, A3-OVA and dgA3-OVA, respectively. In each gel, lane 1 and 2 were before and lane 3 and 4 were after 5 min heating and rapid cooling. Lane 2 and 4 correspond to the samples performed trypsin digestion.

scattering was monitored at wavelength 340 nm in fluorometer (F-3000, Hitachi, Tokyo, Japan) maintaining temperature at 37°C.

Evaluation of the binding of misfolded ovalbumin to GroEL

Binding affinity to GroEL is judged by the competition of the GroEL and DHFR folding intermediate binding. First, 39.1 µg (46.6 pmole) of GroEL was incubated at 15°C in 995 µl of 30 mM Tris-HCl, pH 7.2, 50 mM KCl, 30 mM GdnHCl, 2 mM DTT, 50 µM NADPH, 50 µM DHF with or without 10 µg (222 pmole) of OVA for 5 min. In the experiment to check the system, 5 µl containing 0.2-8 µg (44-220 pmole) of rhodanese (Fig. 6) in 6 M GdnHCl 30 mM Tris-HCl 2 mM DTT incubated 37 °C for 30 min was added to replace for OVA. Then 5 µl of 0.1mg/ml DHFR in 6 M GdnHCl, 30 mM Tris-HCl, pH 7.2, 2 mM DTT incubated for 30 min at 37°C was added and vortexed vigorously. The reaction mixture was transferred to cell incubated at 30°C and the activity of DHFR was monitored from the decrease of NADPH at 3 min from injection of DHFR by measuring absorption of NADPH at 340 nm with spectrophotometer UV-2100s (Shimadzu, Kyoto, Japan). The relative DHFR activity was decided with next equation.

$$\text{Relative DHFR activity} = 100(A_{\text{sample}} - A_{\text{negative}}) / (A_{\text{positive}} - A_{\text{negative}})$$

Where, A_{sample} is the activity observed in the experiment when competitor was added, A_{positive} is the activity when GroEL was not added and A_{negative} is the activity when competitor was added.

Results

Preparation of dephosphorylated and deglycosylated ovalbumin species

The ratio of three species that differ in the numbers of phosphorylation A1:A2:A3 was 85:14.5:0.5 (by ion-exchange chromatography, data not shown) or 85:12:3 (by Native-PAGE, data not shown). The ratio is consistent with that had been reported. The little difference within two methods were to from that the first half fractions of second peak eluted from anion exchange chromatography had separated two bands on Native-PAGE. After ion-exchange chromatography about 500 mg of A3-OVA was purified from 1 g of acid phosphatase treated OVA. And after three steps of purification, 19 mg of dgA3-OVA was purified from 60 mg of A3-OVA.

Measurement of melting temperature

The T_m of each OVA species in salt free and nonreduced condition were

determined from fitting equation for the results of CD ellipticity at 222 nm (Fig. 3), that of A1,A3, dgA3-OVA were 76°C, 74°C, 71°C respectively. These values were lower a little than that from DSC method (Kitabatake *et al.*, 1988), but it was consistent that dephosphorylation decreased the T_m by approximate 2°C in this condition.

Far-UV CD spectra of three ovalbumin species in different condition

Far-UV CD spectra of three OVA species in unheated, heating and rapidly cooled after heating were almost the same in each conditions (Fig. 4). These results suggest that phosphorylation and glycosylation of OVA do not influence on conformation of polypeptide main chain. This suggestion was supported by the result each OVA species before heating had a resistance to trypsin and those after heating had been digested easily.

Salt induced linear polymerization of misfolded ovalbumin

In the correct kinetic model for temperature-induced aggregation of β -lactoglobulin, the size of the scattered light intensity were proportional to the

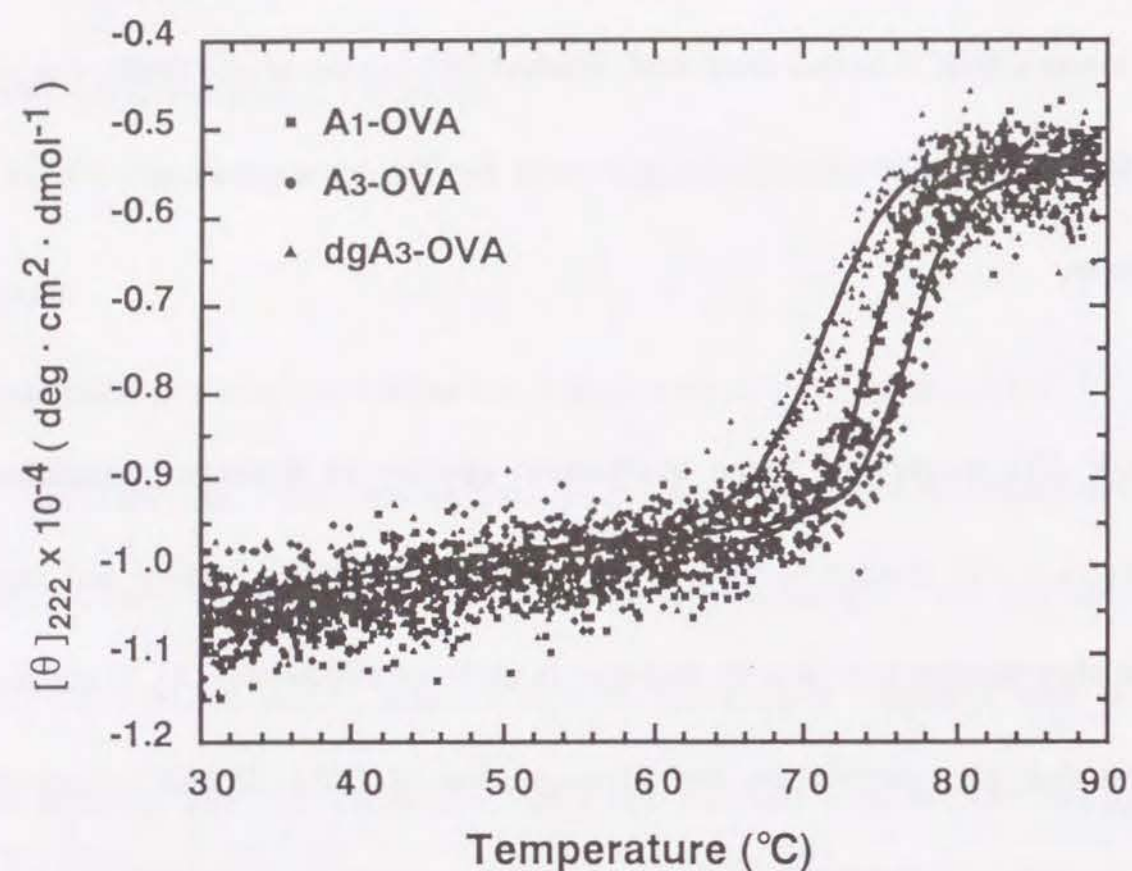


Fig. 3. Thermal denaturation of three ovalbumin species monitored whit CD at 222 nm. In nonreduced conditon, ovalbumin solutions were heated in gradually and monitored the CD $[\theta]_{222 \text{ nm}}$. The intensity of measured were proted by small squares (A1-OVA), circles (A3-OVA), triangles (dgA3-OVA). The melting temperatur (T_m) of three ovalbumin species were determind in the fitting equation as mentioned in methods.

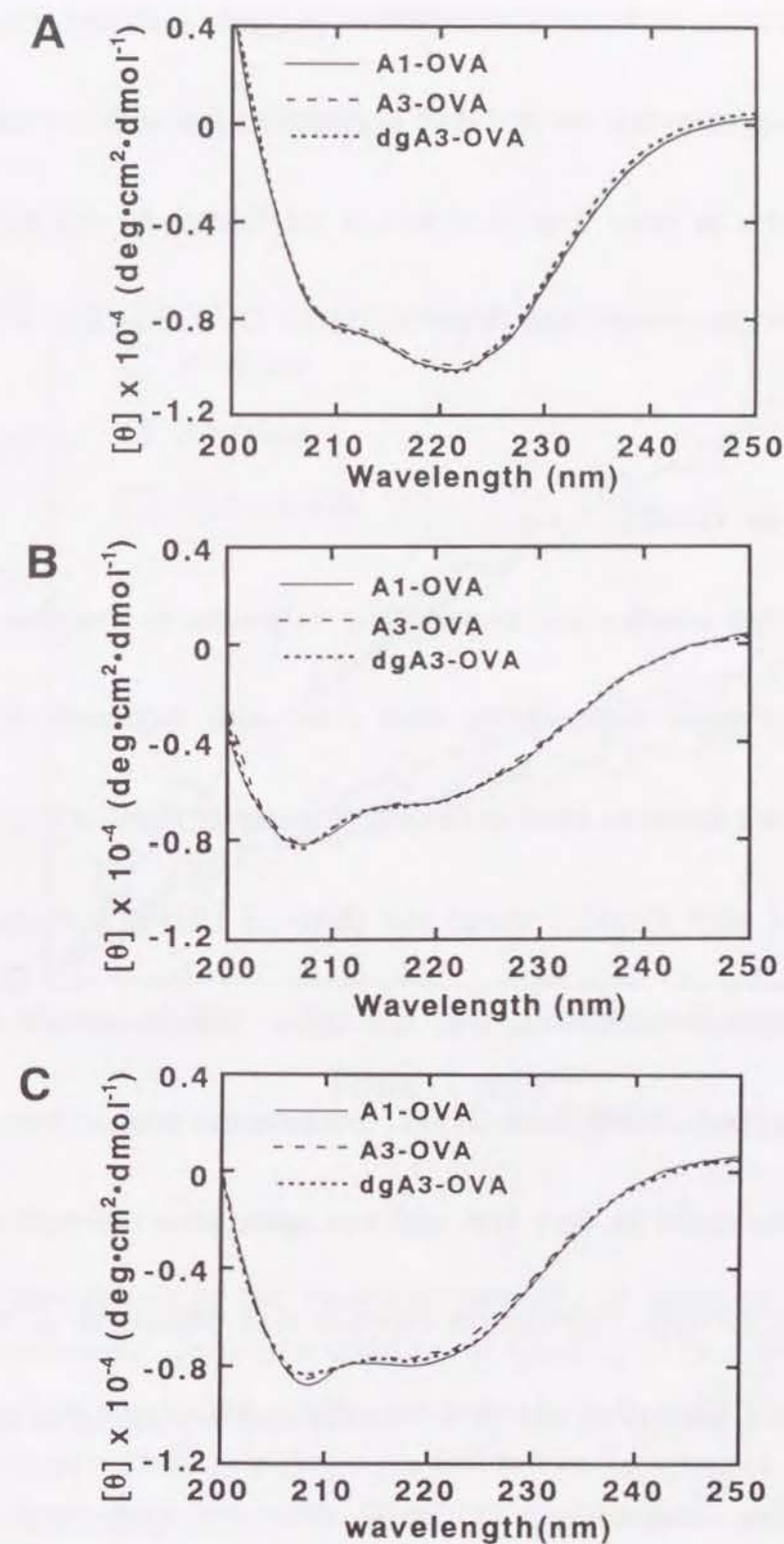


Fig. 4. Far-UV CD spectra of three ovalbumin species in different conditions. The spectra were measured at 0.1 mg/ml protein concentration of three ovalbumin species in different conditions; before heat treatment (A), in heating at 80 °C (B), rapidly cooled after heating 80 °C for 5 min (C).

product of concentration and size of the protein polymer particles. From the initial increase in scattering intensity, swiftness of polymerization within misfolded dgA3-OVA could be faster at least than that within misfolded A1-OVA (Fig. 5). This result means dephosphorylated and deglycosylated OVA was apt to aggregate in misfolded state.

Binding affinity to GroEL

In order to judge whether our experiment is proper to evaluate the binding affinity to GroEL, control experiments were done with rhodanese or casein as a competitor, which are known to bind to GroEL (Langer & Hartl, 1992). In negative control experiment with GroEL, about one third of DHFR activity in positive control experiment without GroEL was remained. This remained activity was attributed to the escaped DHFR from GroEL, because the protein concentrations in the reaction mixtures could be very low, and was agree with reported results. From the relative DHFR activity, calculation formula was described in materials and methods. The relative activity of DHFR were increased depending on the mole ratio of rhodanese folding intermediate to GroEL (Fig. 6). And casein, known to compactly folded but lacks tertiary structure even in physiological condition and to bind weakly to GroEL, was used as a model protein. The value of relative DHFR

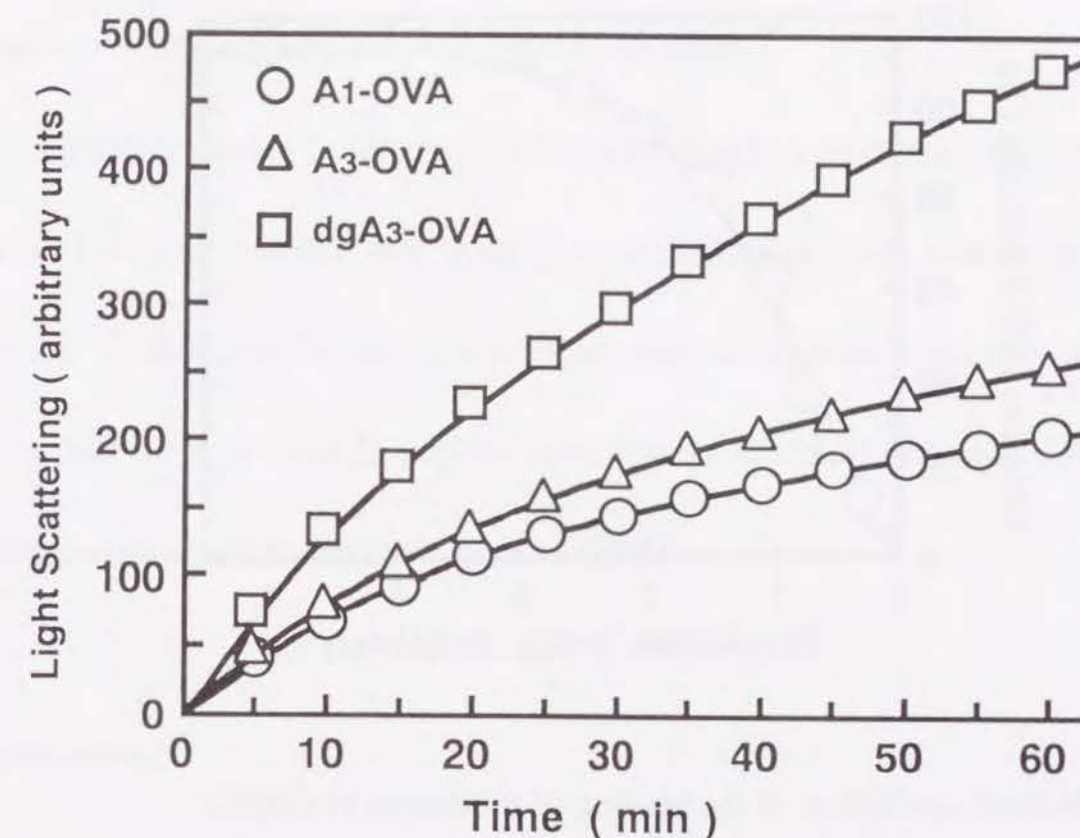


Fig. 5. Comparison of the formation rate of salt induced linear polymers of misfolded ovalbumin. From the addition of Na_2SO_4 at time zero that induced the polymerisation of misfolded ovalbumin in quartz cell at 37°C , the increased light scattering was monitored at wavelength 340 nm in fluorometer. Circles correspond to A1-OVA, triangles corresponds to A3-OVA and squares corresponds to dgA3-OVA.

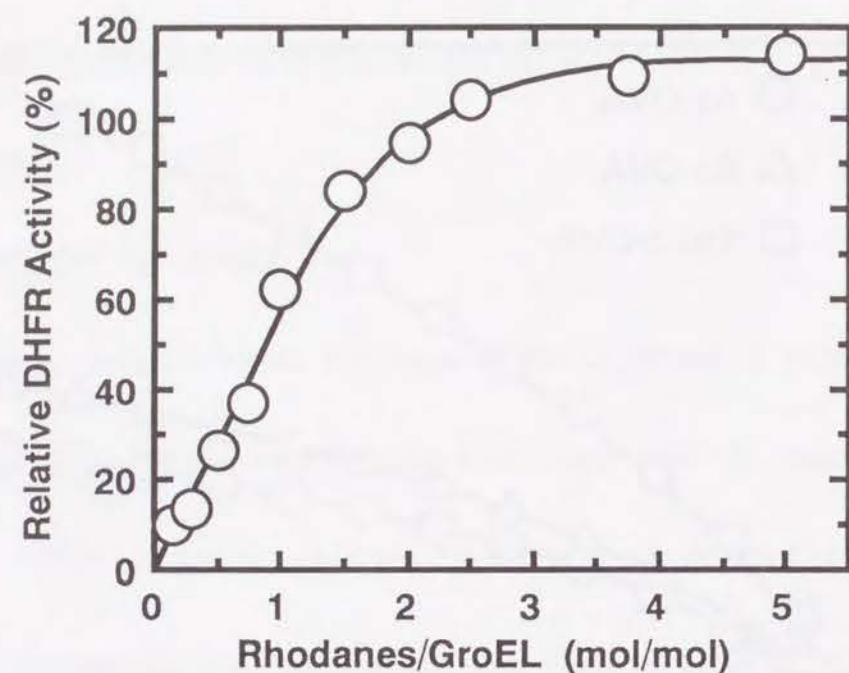


Fig. 6. Indirect evaluation of the binding of rhodanese to GroEL. The molar ratio to GroEL of Rhodanese folding intermediate species from GdmCl denatured state were indicated in the horizontal axis. The relative DHFR activity was calculated with the equation described in methods.

activity in the case of experiment, 5-fold molar excess of casein was added as competitor, was 50% (Fig. 7). Those data were comparable with the results of binding to GroEL performed directly and confirmed our experimental system was proper to evaluate binding of competitor and GroEL.

All OVA species before heated did not bond at all, but all misfolded OVA caused by rapid cooling after heating bonded stronger than casein. In all OVA species, dephosphorylation and deglycosylation increased binding affinity of misfolded OVA to GroEL step by step, that is misfolded OVA that is easier to self-aggregate could bind the stronger to GroEL.

Discussion

Dephosphorylation and deglycosylation of OVA reduce T_m by 2°C and 3°C respectively. There are two cases in reducing the T_m . The first is reduction of ΔG by stabilizing the native conformation or by destabilizing the denatured conformation. The second is reduction of the folding efficiency when the case heat denaturation can assume to be in equilibrium of the two states transition. It takes

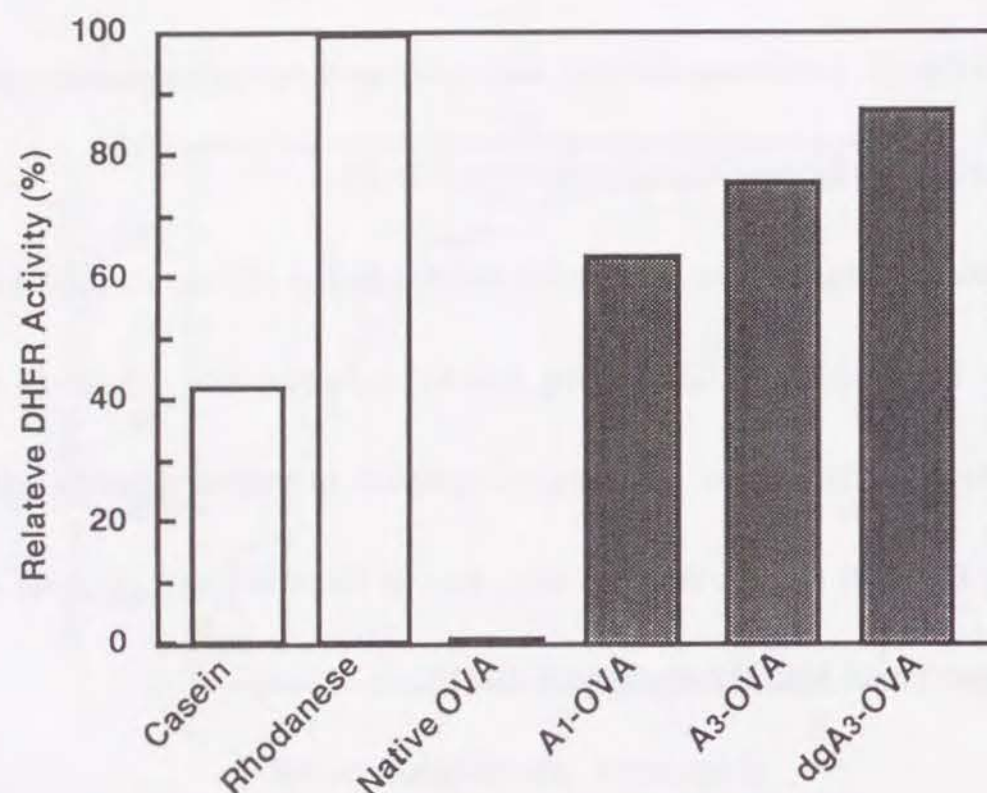


Fig. 7. Indirect evaluation of the binding of misfolded ovalbumin to GroEL. Each column mean the relative DHFR activity observed in the addition of 10-fold molar ratio of indicated samples. Native OVA corresponds to the case of A1-OVA representate three ovalbumin species. Native OVA and casein were added without denaturation process. Rhodanese was completely denatured in 6 M GdmCl. A1-OVA, A3-OVA and dgA3-OVA correspond to the case each heated and rapidly cooled OVA species was added as a competitor.

so long time to refold from heat denatured state, even in A1-OVA only less than 20% of OVA refold completely in 60 min at 75°C. From this point the reason of reduction of T_m could be second case.

There is few report that investigated about the phosphoryl groups on the nonspecific aggregation of misfolded polypeptide, though so many information have been known on the specific effect on inter molecular polymerization; for example in the case of myosin filaments elongation(Kuczmarski *et al.*, 1987). But some reports about the example in which carbohydrate moiety prevent the loss of enzyme activity from aggregation. The reasons would come from three points; 1) stabilizing the native structure 2) increasing the efficiency of refolding from misfolded state 3) decreasing the affinity between the misfolded molecules. In this study, the experiment has an advantage, that is we could investigate the affinity between the misfolded molecules. Since the rate of polymerization in this salt induced polymerization would not effect from 1) and 2) in the very early period. Roefs *et al.* substantiated that the scattered light intensity is proportional to the product of concentration and size of the polymer particles. From the initial increase in scattering intensity observed in misfolded A3-OVA and dgA3-OVA, carbohydrate moieties of ovalbumin would decreased the affinity between

misfolded molecules but phosphoryl groups has little effect. In all OVA species, dephosphorylation and deglycosylation increased binding affinity of misfolded OVA to GroEL step by step, that is misfold OVA that the easier to selfaggregate could bind the stronger to GroEL. Since, GroEL recognize the denatured protein exposing the hydrophobic clusters, the results suggest that misfolded OVA has the hydrophobic clusters and the phosphoryl and carbohydrate moiety of OVA inhibit the association of the hydrophobic clusters to another molecules. These data are comparative to that the *N*-linked sugars carried on invertase inhibit the interaction with GroEL during folding from chemical denaturation (Kern *et al.*, 1992).

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SUMMARY

In CHAPTER 1

The folding of heat-denatured ovalbumin, a member of the serpin superfamily with a molecular size of 45 kDa, was examined. Ovalbumin was heat-denatured at 80°C under non-reducing conditions at pH 7.5 and then cooled either slowly or rapidly. Slow cooling allowed the heat-denatured ovalbumin to refold to its native state with subsequent resistance to digestion by trypsin. This result suggested that, with slow cooling, heat denaturation is a reversible transition that is under thermodynamic control. Upon rapid cooling, by contrast, the heat-denatured ovalbumin became trapped in a metastable non-native state that was susceptible to trypsin. The non-native state was marginally stable for several days at a low temperature, but the protein was transformed slowly into the native form. Considering data from size-exclusion chromatography and from analyses of circular dichroism, intrinsic tryptophan fluorescence and adsorption of 1-anilino-naphthalene-8-sulfonate, I postulated that the non-native state that accumulated upon rapid cooling is a compact intermediate state with disordered side chains, similar to the molten globule state. The temperature dependence of the refolding kinetics indicated that the yield of renatured protein was maximal at about 55°C. The refolding kinetics were biphasic, with the amplitude of a rapid phase increasing

with decreases in temperature, and with a concomitant decrease in the amplitude and rate of a slow phase. These findings suggested the kinetic partitioning between alternative folding pathways: one that directed the protein slowly toward the native state and the other that led rapidly to a molten globule state. Analysis of disulfide pairing revealed the formation of a scrambled form with non-native disulfide interactions in both the heat-denatured state and the molten globule state that accumulated upon rapid cooling, suggesting that non-native disulfide pairing is responsible for the kinetic barriers that retard the correct folding of ovalbumin. The non-native species, which were in a large variety of non-specific compact conformations, were considered to be trapped as metastable intermediates in a kinetically accessible state. Thus, the details of the correct folding of heat-denatured ovalbumin indicate the presence of alternative pathways and support a recent hypothesis of multiple folding pathways with a rugged energy landscape.

In CHAPTER 2

Polymerization caused by a defective folding of heat-denatured ovalbumin was examined. A compactly misfolded ovalbumin that was produced by cooling heat-denatured protein rapidly had a high tendency to aggregate in the presence of salt. Two different forms of aggregates were observed with varying concentrations of a salt: a linear polymer at a physiological concentration and a massive

agglomerate at a higher concentration. Salt-induced polymerization depended preferentially on species of anions in the order of effectiveness which followed the lyotropic series of Hofmeister. A defective folding of heat-denatured ovalbumin induced the exposure of cysteine residues in the sequences that located in the interior of the native protein. The misfolded ovalbumin, but not the native protein, bound to bovine BiP and stimulated its ATPase activity with the K_m of 64 μM and the V_{max} of 0.5 nmol/min per milligram. Measurement of surface plasmon resonance revealed that only the misfolded ovalbumin was recognized with the K_d of 4.12×10^{-8} M by the Fab fragment of monoclonal antibody, which was raised against hen ovalbumin and its epitope was determined to be a hydrophobic segment in the β -strand of central sheet A. Transmission electron microscopy showed that the linear polymerization was inhibited by the addition of bovine BiP and the Fab fragment. These results demonstrated that the compactly misfolded ovalbumin polymerized through hydrophobic interaction occurring among the areas exposed as a result of a defective folding of heat-denatured protein. Exposure of the region of, or adjacent to, the central β -sheet A was required for axial contact among the misfolded molecules, suggesting that this process may be explained by reference to the mechanism speculated for loop-sheet polymerization as was observed in the Z type variant of a serpin α_1 -antitrypsin.

The effects of phosphoryl groups and carbohydrate moiety were studied on inhibition of aggregation in misfolded ovalbumin (OVA). Most ovalbumin purified from hen eggs is A1-OVA that has two phosphoryl groups and a N-linked sugar, and has its melting temperature (T_m) at 76°C. The T_m of A3-OVA, dephosphorylated species, and the T_m of dgA3-OVA, deglycosylated from A3-OVA enzymatically were 74°C and 71°C respectively. From far-UV circular dichroic spectra, three ovalbumin species in unheated, heating and rapidly cooled after heating were almost the same in each condition. It had been founded ovalbumin comes to misfolded state in rapid cooling after heating without salt condition and these misfolded ovalbumin could be induced polymerization with salt addition. Then the increase in scattered intensity after addition of salt to misfolded ovalbumin of three species were measured that means polymerization of misfolded ovalbumin. Formation of selfaggragation was slightly faster in A3-OVA and especially faster in dgA3-OVA. On the other hand, the affinity to chaperonin in *E.coli* (GroEL), that is known to bind to misfolded polypeptide and inhibits their aggregation, were the higher to the ovalbumin species which were more selfaggregatable. These results suggested that phosphoryl groups and carbohydrate moiety of ovalbumin stabilize the conformation in unheated state and play chaperone like role to inhibit aggregation in misfolded state.

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